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(54) Title: AGROBACTERIUM TUMEFACIENS RPOA GENE

(57) Abstract

The present invention is directed to a method for expression of at least one heterologous gene in a host cell comprising transforming a host cell with at least one nucleic acid construct comprising a complete α subunit of an RNA polymerase or a portion thereof of a hybrid nucleic acid containing a portion of the α subunit of an RNA polymerase obtained from the same genus as the heterologous gene, and transforming the host cell, with at least one heterologous gene; and culturing the transformed host cell. The present invention further is directed to nucleic acid molecules used in the present method, vectors containing these nucleic acid molecules, and host cells containing the nucleic acid molecules. The nucleic acid encoding the α subunit of an *Agrobacterium* RNA polymerase and the corresponding amino acid sequence and portions thereof is disclosed.

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AGROBACTERIUM TUMEFACIENS RPOA GENE

This application is a continuation-in-part of provisional application Serial No. 60/129,682 filed on April 16, 1999 and a continuation-in-part of provisional application Serial No. 60/134,206 filed on May 14, 1999, both of which are incorporated herein in their entirety by reference.

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The present invention relates to nucleic acid molecules and constructs containing these nucleic acid molecules and methods of using these constructs to express heterologous genes in hosts. In particular, the nucleic acid molecules of the present invention comprise a DNA sequence encoding at least a portion of the α subunit of the RNA polymerase (referred to herein as RNAP) obtained from the same genus source from which the heterologous genes were originally isolated. The nucleic acid constructs also can optionally comprise, if required for expression of the heterologous genes, at least one gene encoding a transcriptional regulator, also obtained from the same genus as the source of the heterologous genes.

BACKGROUND OF THE INVENTION

Although many host cell systems are well characterized and utilized to express a number of heterologous genes, there are still many genes that cannot be expressed in such host cell systems, as for example, *Escherichia coli* (*E. coli*). It is believed that the lack of expression in these host cell systems is a result of the promoter sequences of the heterologous genes not being recognized by the host RNAP. The present invention has solved this problem by showing that this block in expression can be overcome by co-expression of at least a portion of the *rpoA* gene product, the gene that encodes the α subunit of the RNAP, obtained from the same genus as the source of the heterologous gene that is desired to be expressed in the new host cell. The co-expressed α subunit of the RNAP from the same genus as the source of the heterologous gene combines with the other

subunits of the RNAP, β, β', and σ, to form a functional RNAP. Additionally, if transcriptional regulators, such as transcriptional activators or transcriptional repressors, or additional other subunits of the RNAP, such as β, β' or σ, are required to obtain expression of the heterologous gene, then these additional components also are obtained from the same genus as the source of the heterologous gene. Since two different *rpoA* genes are present in a single host cell, there will be various combinations of RNAPs present in the host cell (1) RNAP containing two α subunits of the same genus as the host (2) RNAP containing two α subunits from the same genus source as the heterologous gene, and (3) RNAP containing one α subunit from the same genus as the host and one α subunit from the same genus source as the heterologous gene. A heterologous gene is intended to mean a gene that is not from the same source as the host cell.

Agrobacterium tumefaciens is a Gram-negative soil bacterium which is the causative agent of Crown Gall disease, affecting primarily dicotyledonous plant species (reviewed in 18, 62). The pathogen incites production of the characteristic tumor through the transfer of a piece of DNA (T-DNA) from the Ti (Tumor inducing) plasmid into susceptible plant cells, with subsequent integration into the host genome. The T-DNA contains genes that direct the biosynthesis of auxin and cytokinin in infected cells (1, 57), resulting in uncontrolled cell division leading to production of the characteristic tumor. The T-DNA also contains genes for the biosynthesis of unique compounds called opines which the bacterium can utilize as a carbon and nitrogen source (39).

Successful transfer of the T-DNA is dependent on the coordinated expression of virulence *(vir)* genes located on the Ti plasmid but separate from the T-DNA. Expression of *vir* genes occurs in response to certain phenolic compounds released from wounded plants (54). This expression is augmented by monosaccharides *(5,* 52), and an acidic pH (38) which are characteristics of plant wound sites. Expression of *vir* genes requires *virA* and *virG,* which are members of the family of two component regulatory systems (60). VirA is an inner membrane associated histidine protein kinase which autophosphorylates in response to the environmental signals (19, 28). The phosphate moiety is subsequently transferred to the aspartate residue of VirG, which in turn activates transcription from promoters containing a specific 12 base pair sequence called the *vir* box, present in the promoters of all *vir* genes (29, 44). In addition to *virA* and *virG*, other chromosomally

encoded genes have been identified in *A. tumefaciens* that have been shown to modulate virulence gene expression either directly or indirectly (12, 15, 20, 61).

The use of *E. coil* as a heterologous host in which to study the regulation of *A. tumefaciens* virulence genes and the mechanism of T-DNA transfer constitutes an ideal model system given the degree of characterization at both the biochemical and genetic level. However, all previous attempts to reconstitute *vir* gene expression in *E. coil* have not been successful. Possible explanations for the lack of *vir* gene expression include the presence of unidentified regulatory genes in *A. tumefaciens* required for *vir* induction, and/or that *E. coli* may contain specific repressor(s) of *vir* gene induction.

A characteristic of *vir* gene promoters is the absence of a strong -35 sequence (10). Dnase I footprinting studies have shown that VirG protects a region extending into where the -35 consensus sequence should be (29, 44). It has been suggested that binding of VirG may functionally replace the -35 consensus sequence allowing transcription to occur. This situation is similar to Class II CAP-dependent promoters, in which the CAP binding site overlaps with the -35 sequence. Studies have demonstrated that transcription at Class II CAP-dependent promoters requires interaction between CAP and the α subunit (*rpoA*) of RNAP (42, 49, 64). Many transcriptional factors from *E. coli* are known to require interaction with the α subunit of RNAP , including FNR (59), GaiR (9), MarA (24), Mer R (7), MetR (23), OxyR (56), OmpR (21), Rob (26), SoxS (25), and TyrR (34). Analysis of the α subunit from *E. coli* indicates the presence of two independent domains, the N-terminal domain and the C-terminal domain (21, 27, 63). The N-terminal domain (NTD) is involved in the assembly of the core polymerase, while the C-terminal domain (CTD) is involved in interaction with certain transcriptional regulators (7, 9, 21, 23, 24, 25, 26, 32, 34, 47, 56, 59). Recently, interaction between the NTD and CAP at Class II CAP-dependent promoters has been demonstrated (42,49).

The present methods of expressing heterologous genes are particularly useful to express multiple genes or operons in a metabolic pathway by co-expressing the *rpoA* gene product obtained from the same natural hosts from which the genes or operons were originally isolated with the multiple genes. The present method provides an advantage over existing multiple gene expression systems by eliminating the need to separately isolate each gene in the metabolic pathyway and link each gene to a promoter that is functional in the host. Expression of heterologous genes for an entire metabolic pathway in hosts is

particularly useful to produce particular metabolites, such as pharmaceuticals, food supplements, chemical products or fine chemical products.

The present invention further provides nucleic acid molecules and methods whereby heterologous genes encoding metabolites in an entire metabolic pathway can be introduced into hosts so that these hosts possess specific catabolic and/or metabolic characteristics that allow the hosts to grow or express gene products under specific environmental conditions. These hosts are useful in bioremediation, bioassays and biocontrol studies where manipulation of natural or symbiotic bacterial flora is desired, such as in the control of parasites or insects where bacterial symbiants are involved.

The present invention broadly relates to the expression of heterologous genes in a host using nucleic acid molecules comprising a gene encoding the complete α subunit of the RNAP obtained from the same genus as the source of the heterologous genes or at least a portion of the α subunit of the RNAP. The host and the heterologous genes to be expressed may be prokaryotic or eukaryotic in origin, however, prokaryotic hosts are preferred. The present invention also relates to a hybrid nucleic acid molecule encoding a hybrid α subunit of RNAP, where a first nucleic acid comprises at least a portion of the *rpoA* gene encoding the α subunit of an RNAP obtained from the same genus as the source of the host cell and a second nucleic acid comprises at least a portion of the α subunit of an RNAP obtained from the same genus as the heterologous gene.

The present invention specifically discloses the *rpoA* gene from *Agrobacterium* encoding the α subunit of the RNAP and the corresponding amino acid sequence. Portions of both the *rpo*A gene and the α subunit of RNAP of *Agrobacterium* also are encompassed by the present invention, particularly the sequences disclosed in Figs. 2A and 2B. The *rpo*A gene is useful in methods of expressing *Agrobacterium* genes in hosts, particularly prokaryotic host cells. Prior to the present invention, it was not possible to express *Agrobacterium* genes, particularly *Agrobacterium* virulence genes, in *E. coli*. Therefore, the present invention provides a method for studying *Agrobacterium* genes and their regulation.

The invention also relates to genes encoding hybrid *rpoA* genes that comprise at least a portion of an *rpoA* gene from the same genus as a heterologous gene which is expressed, such as the *Agrobacterium rpoA* gene, with the remainder of the *rpoA* gene obtained from the same genus as the host cell in which the heterologous gene is expressed.

SUMMARY OF THE INVENTION

The present invention is directed to an isolated nucleic acid molecule encoding the complete α subunit of an RNAP of *Agrobacterium* or at least a portion thereof. Specifically, the present invention is directed to an isolated nucleic acid molecule encoding the complete α subunit of an RNAP of *Agrobacterium*, in which the amino acid sequence of the α subunit is depicted in Figure 2A or at least a portion of this sequence. More specifically, the present invention is directed to an isolated nucleic acid molecule comprising the complete nucleic acid sequence as depicted in Figure 2B or at least a portion thereof. These isolated nucleic acid molecules encoding at least a portion of the α subunit of an RNAP of *Agrobacterium* also are optionally linked to a promoter that is functional in the host in which these molecules are expressed. Further, the present invention is directed to vectors containing these nucleic acid sequences or molecules. The vectors are useful in transforming host cells for the expression of heterologous gene(s). The invention is also directed to host cells that are transformed with the nucleic acid molecules described herein. The nucleic acid molecules useful to transform the host cells are operably linked to a promoter and encode an α subunit of an RNAP or at least one heterologous protein.

The present invention is also directed to a hybrid nucleic acid molecule for the expression of at least one heterologous gene in a host cell comprising a first nucleic acid sequence encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the host cell and a second nucleic acid sequence encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the source of the heterologous gene; and vectors containing the hybrid nucleic acid molecule.

The present invention is further directed to a method of expressing at least one heterologous gene in a host cell comprising transforming a host cell with a vector comprising a nucleic acid molecule encoding a complete α subunit of RNAP from the same genus as the source of the heterologous gene or at least a portion thereof, and also transforming the host cell with a vector comprising at least one heterolgous gene. The nucleic acid molecule and the heterologous gene(s) also may be contained in one vector but preferably these sequences are contained within two or more vectors. In a further embodiment, the present invention is also directed to a method of expressing at least one heterologous gene in a host cell comprising transforming a host cell with a vector comprising a hybrid nucleic acid molecule comprising a first nucleic acid sequence

encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the host cell and a second nucleic acid sequence encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the source of the heterologous gene, and transforming the host cell with a vector comprising at least one heterologous gene; and culturing the transformed host cell under conditions where the heterologous gene is expressed in the host cell. The host cell also may be transformed with a single vector containing the nucleic acid molecule encoding the complete α subunit of the RNAP, a portion thereof or a hybrid molecule comprising a portion of the α subunit of the RNAP, and with the heterolgous gene(s) or α subunit of the RNAP peferably, two or more vectors may be used. The nucleic acid molecules encoding the α subunit and the heterologous gene(s) may be in separate vectors for transformation of the host cell. The host cell can optionally be transformed with at least one gene encoding a heterologous transcriptional regulator obtained from the same genus as the source of the heterologous gene. The heterologous gene may comprise multiple genes or operons in the same metabolic pathway.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Physical and genetic map of recombinant plasmids carrying the rpoA gene from *A. tumefaciens* is depicted. Abbreviations: B, BamHI; E. FcoRI; P, PstI; Sa, Sm3A; St, Sttd, X, XhoI Location and direction of *rpoA* and *lac* promoter for constructs pPS1.3R and pPS1.3 are indicated by arrows.

Figure 2A. The amino acid sequence of the *A. tumefaciens* α subunit of the RNAP is depicted.

Figure 2B. The *A. tumefaciens rpoA* gene encoding the *A. tumefaciens* α subunit of the RNAP is depicted. The actual coding sequence for the α subunit is located between nucleotide 355 (atg) and nucleotide 1356 (taa).

Figure 3. Amino acid sequence alignment of the *A. tumefaciens and* E. coli rpoA gene products. Identical amino acid residues at a given position are marked by an asterisk, and conserved substitutions are marked by a dot. Gaps introduced to allow optimal alignment are designated by a dash. The bolded nucleotides show three highly conserved regions between the two amino acid sequences.

Figure 4 A and B. Assembly of His-rpoA into RNAP, and *in vitro* transcription assays. Figure 4A - SDS-PAGE analysis of reconstituted RNAP containing *E. coli* β, β',

σ^{70} subunits and His-RpoA from *E. coli* or *A. tumefaciens* and stained with Coomassie brilliant blueB. Figure 4B - *In vitro* transcription from *virB* and *galP*1 promoters in the absence and presence of $VirG^{con}$. The figure shows the result of a multiround transcription reaction employing reconstituted RNAP containing His-rpoA from either *E. coli* or *A. tumefaciens*. Where indicated, 2 pmol of $VirG^{con}$ was added to the reaction.

Figure 5. Protein-protein and protein-DNA interactions between RpoA, $VirG^{con}$ and the *virB* promoter. The figures show electrophoretic mobility shift assays of [^{32}P]-labeled *virB* promoter containing purified $VirG^{con}$ and His-RpoA from *E. coli* in Figure 5A or *A. tumefaciens* in Figure 5B Lane 1: Promoter only, Lane 2: Promoter + $VirG^{con}$, Lane 3: Promoter + 150 nM RpoA, Lane 4: Promoter + 300 nM RpoA, Lane 5: Promoter + 600 nM RpoA, Lane 6: Promoter + $VirG^{con}$ + 150 nM RpoA, Lane 7: Promoter + $VirG^{con}$ + 300 nM RpoA, Lane 8: Promoter + $VirG^{con}$ + 600 nM RpoA.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Although many well characterized host cells, such as *E. coli*, provide advantages as an expression host cell, it has not been possible to express the genes of many bacteria in these host cells because the promoter sequences of the heterologous genes are not recognized by the host cell RNAP. The present invention provides a method of overcoming this block to expression by co-expressing the α subunit of the RNAP from the same genus as the source of the heterologous genes that are to be expressed in the host,and optionally co-expressing the transcriptional activator from the same genus as the source of the heterologous genes. The success of this method has been demonstrated by showing the expression of the *Agrobacterium tumefaciens* virulence (*vir*) gene operons in *E. coli*. In this specific example, expression of these genes was shown to require not only the *vir* gene specific transcriptional regulator but also the *rpoA* gene from *A. tumefaciens*. The *A. tumefaciens* α subunit of the RNAP is able to assemble with the other subunits (β, β' and σ subunits) of the *E. coli* RNAP to form a functional polymerase which specifically recognizes the *Agrobacterium* genes in the presence of the *Agrobacterium* transcriptional regulator, specifically an activator. The results show that this interaction is genus specific, in that, the transcriptional activator from *Agrobacterium* can only efficiently interact with the rpoA gene product from *Agrobacterium* and not with that of *E. coli*.

Conventional understanding of gene expression in prokaryotes emphasizes the importance of the transcriptional regulator, either an activator or a repressor. A transcriptional activator is a protein that is needed to turn on the expression of target genes either directly or indirectly whereas repressors are proteins that inhibit the transcription of target genes either directly or indirectly. In the present invention, it is shown that the α subunit of the RNAP is the most critical factor in activating bacterial genes, especially those requiring transcriptional regulators. For example, to express heterologous genes or operons from organism A in organism B, at least a portion of the *rpoA* gene from organism A is also needed to be co-expressed in organism B. Likewise if transcriptional regulators are required for expression of heterologous genes, the genes encoding the transcriptional regulator(s) from organism A should also be used to transform the host because these regulators can most efficiently interact with RpoA from the same organism A but not as well or at all with that of organism B.

The present method is particularly advantageous when it is used to express particular heterologous metabolic pathway genes in hosts for bioremediation/biocontrol purposes or production of particular metabolites. This approach becomes much more practical than fusing each gene or operon under the control of a known promoter that is functional in the host.

Expression of genes for an entire metabolic pathway in heterologous hosts is useful to produce particular metabolites or to confer to the host specific catabolic or metabolic characteristics that now enables the host to grow or express gene products under specific environmental conditions.

An example of the utility of the present method is shown by the production of a plant growth promoting molecule, indole-3-acetic acid, from *Azospirillum brasilense*, that requires at least 3 biosynthetic genes. *A. brasilense* also possesses multiple genes that encode products that fix nitrogen (65). The multiple genes responsible for encoding these products and the *rpoA* gene are introduced via one or more vehicles, such as plasmids or cosmids, into a host that is applied to a plant and that expresses the products of the multiple genes. The expressed plant growth promoting molecules and nitrogen fixing molecules affect the growth of the treated plant.

Further *Rhizobium*, *Bradyrhizobium*, *Azorhiobium* and *Sinorhizobium* are other examples of nitrogen-fixing bacteria, each involving at least two dozen genes for nodulation

(symbiosis) and multiple genes for nitrogen fixation. These bacteria, as well as other bacteria that produce useful products for plants, may also be the source of the heterologous genes and the *rpoA* gene.

The present invention can also be utilized to express gene product(s) in a catabolic pathway that degrades or breaks down toxic or environmentally hazardous products. For example, a *Pseudomonas* species strain ADP expresses at least four genes that are involved in the degradation of a herbicide, Atrazine (66). These genes are introduced and expressed in a host by applying the host to areas where this herbicide is present, resulting in the degradation of the herbicide on site.

Further, the present invention is useful in providing a biocontrol system that includes a host that expresses heterologous gene products, that for example, kill disease producing organisms on a plant, animal, human or a surface that will support growth of the host. *Pseudomonas chloroaphis* strain 30-84 expresses at least three antifungal metabolites, phenazine-1-carboxylate, 2-hydroxyphenazine-1-carboxlylic acid and 2-hydroxyphenazine that involves four biosynthetic genes and 2 regulatory genes (67). These genes may be introduced into a plant bacteria, such as *Rhizobium*, that can be applied to a plant to control the growth of fungi on the plant.

The present invention also is useful as an *A. tumefaciens* gene delivery system in heterologous hosts for potential gene delivery or gene therapy systems of *A. tumefaciens* genes to various hosts.

The present invention also can be used to express heterologous genes in *A. tumefaciens* to alter its characteristics (e.g. host specificity) for a broader application of the *A. tumefaciens* mediated gene delivery system.

The present invention also is useful to prepare host cells containing an *rpoA* gene that is heterologous to the host cell, such as Escherichia. This host cell is useful for studying changes in gene expression patterns in this cell.

The present invention can additionally be used to express particular sets of heterologous genes, including multigene operons/stimulons or genes encoding multi-subunit protein complexes, such as proteosomes or gene libraries in hosts as targets for drug screening and/or diagnostics.

The present invention discloses an isolated nucleic acid molecule encoding the complete α subunit of an RNAP of *Agrobacterium* or at least a portion thereof. More

specifically, an isolated nucleic acid molecule encoding the complete α subunit of an RNAP of *Agrobacterium*, where the amino acid sequence of the α subunit is depicted in Figure 2A is disclosed or at least a portion thereof. The portion can be a length of amino acid sequences that is sufficient to express any heterologous gene in the host cell into which it is introduced. The portion of the α subunit of the RNAP also should be of a sufficient length to provide at least one of the following functions: 1) bind to DNA in the promoter region 2) interacts with the other subunits of RNAP; i.e., β, β' and σ to form a functional RNAP, and 3) interacts with the transcriptional activator. More specifically, an isolated nucleic acid molecule comprising at least a portion of the nucleic acid sequence as depicted in Figure 2B and that encodes at least a portion of the amino acid sequence of Figure 2A is disclosed. The nucleic acid molecule (rpoA) encoding the *Agrobacterium* α subunit of the RNAP or a portion of the nucleic acid molecule is useful in the expression of *Agrobacterium* genes in any prokaryotic host cells.

The present invention is also directed to a nucleic acid molecule encoding a hybrid α subunit of RNAP where the isolated nucleic acid molecule is inserted in a vector and is useful in expressing at least one heterologous gene in a host cell comprising a first nucleic acid sequence encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the host cell and a second nucleic acid sequence encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the source of the heterologous gene. This isolated nucleic acid molecule is operably linked to a promoter so that it is under the control of a promoter that is functional in the host cell. For example, the *E. coli lac* or *trp* promoters are useful to express the *rpo*A gene in an *E. coli* host cell. Promoters that function in numerous host cells are well known to persons skilled in the art and can be selected based upon the host cell selected. The second nucleic acid sequence is obtained from any eukaryotic or prokaryotic host cell, and more particularly from a prokaryotic cell, including Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria. More specifically, the bacteria are selected from the genus *Agrobacterium*, *Mycobacterium*, *Chlamydia*, *Pseudomonas* or *Streptomyces*. More preferably, it is obtained from *Agrobacterium*, and most preferably the second nucleic acid sequence encodes the complete amino acid sequence as depicted in Figure 2A or a portion thereof, with the sufficient length of this portion discussed above. The portion of the of the *rpoA* gene can be from

any position in the gene; i.e., that is from the 5' or the 3' end of the gene, which encodes the N-terminus and C-terminus of the α-subunit, respectively. Most preferably, the nucleic acid molecule portion encodes at least amino acid residues 157 to 336 as depicted in Figure 2A. The nucleic acid molecule can encode as few as 8 consecutive amino acid residues of any portion of Figure 2A. Specifically, a portion comprising at least the amino acid residues 157 to 336, or of less than amino acid residues of 157 to 336, and more specifically amino acid residues 329 to 336 are useful in the present invention. The first nucleic acid sequence is obtained from any bacteria but more preferably from the genus *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*.

The present invention is directed to a method of expressing at least one heterologous gene in a host cell comprising transforming a host cell with a nucleic acid construct comprising a first nucleic acid sequence encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the source of the host cell, and a second nucleic acid sequence encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the source of the heterologous gene; and with at least one heterologous gene; and culturing the transformed host cell under conditions wherein the heterologous gene is expressed in the host cell. The method further comprising transforming the host cell with at least one gene encoding a heterologous transcriptional regulator obtained from the same genus as the source of the heterologous gene. The heterologous gene may comprise multiple genes or operons in the same metabolic pathway that catalyze the production of a product.

The host cells useful in the present method are prokaryotic cells or eukaryotic cells. Preferably, the host cell is selected from a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, aerobic bacteria, acid-fast bacteria, mycoplasma, anaerobic bacteria, and facultative bacteria. Preferably, the host cell is obtained from the genus *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*. Eukaryotic cells may be selected from known mammalian cells that can be grown in cell culture, such as CHO, BHK, COS.

The present invention also is directed to a method of expressing at least one heterologous gene in a host cell comprising transforming the host cell with a vector comprising a nucleic acid molecule encoding a complete α subunit of an RNA polymerase obtained from the same genus as the heterologous gene or a portion of the α subunit of the

RNA polymerase, and also transforming the host cell with at least one vector comprising at least one heterologous gene; and then culturing the transformed host cell under conditions where at least one of the heterologous genes is expressed in the host cell. The method further comprises transforming the host cell with at least one gene encoding a heterologous transcriptional regulator obtained from the same genus as the source of the heterologous gene. The transcirptional regulator is a transcriptional activator that interacts with the α subunit of the RNAP to enhance the expression of the heterologous gene. The heterologous gene may comprise multiple genes or operons in the same metabolic pathway.

Preferably, the host cell is transformed with two vectors, one containing the *rpoA* gene or a portion thereof and one containing at least one heterologous gene. However, the host cell may be transformed with a single vector containing the *rpoA* and heterologous genes, but more than two vectors can be used to introduce the genes into the host cell depending on the number of genes and the size of the nucleic acid to be introduced.

The host cell may be a prokaryotic cell or a eukaryotic cell. Preferably the host cell is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria. More preferably the host cell is from the genus *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*. Most preferably the host cell is an *Escherichia*.

The method is useful for the expression of at least one heterologous gene, where the genus of the source of the heterolgous gene is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, aerobic bacteria, anaerobic bacteria, and facultative bacteria. More preferably the genus of the source of the heterologous gene is *Agrobacterium*, *Mycobacterium*, *Chlamydia*, *Pseudomonas* or *Streptomyces*, and most preferably, the genus is obtained from *Agrobacterium*.

The method utilizes a nucleic acid molecule that encodes the α subunit of the RNAP the amino acid sequence as depicted in Figure 2A or at least a portion of the amino acid sequence depicted in Figure 2A. The portion of the amino acid sequence useful in the present method may be from the N-terminus or C-terminus of the α subunit of the RNAP but preferably comprises at least amino acid residues 157 to 336 of the amino acid sequence depicted in Figure 2A or the portion of the amino acid sequence may comprise less than amino acid residues 157 to 336 of the amino acid sequence depicted in Figure 2A. The

portion of the amino acid sequence should comprises at least 8 consecutive amino acid residues of Figure 2A. More specifically, the portion of the amino acid sequence as depicted in Figure 2A comprises amino acid residues 329 to 336.

The present invention also encompases a purified α subunit of an RNAP of *Agrobacterium*. More specifically, the purified α subunit of an RNAP of *Agrobacterium* of the present invention is the amino acid sequence depicted in Figure 2A. The invention is also intended to encompass portions of the purified α subunit of an RNAP disclosed in Figure 2A. More specifically, the purified α subunit of an RNA polymerase of *Agrobacterium* or a portion thereof is encoded by the nucleic acid is depicted in Figure 2B or a portion thereof.

A *rpoA* gene from the same genus as the source of a heterologous gene encoding the α subunit from this source is obtained by methods well known to persons skilled in the art. For example, this gene can be obtained: (1) by complementation of the a temperature sensitive *rpoA* mutant strain of a known organism, i.e. E.coli as discussed below in the preferred embodiment; (2) by Southern hybridization based on DNA sequence homology; (3) by Western blot based on cross reactivity of an antibody against a known RNAP α subunit; and (4) by PCR amplification based on conserved DNA sequences among known rpoA genes. For their expression, the *rpoA* genes are fused behind or operably linked to a promoter that is known to be operable in the host. The fusion is either a transcriptional fusion or translational fusion. Many standard methods are well known to persons skilled in the art and can be utilized to carryout these methods (See reference 68 which is herein incorporated in its entirety by reference.)

The following specific examples set forth below serve to further illustrate the present invention in its preferred embodiments, and are not intended to limit the present invention to these examples.

EXAMPLES

Bacterial strains, plasmids and media.

All strains and plasmids used or constructed are listed in Table 1. Bacterial strains were grown in either LB medium (40), Mannitol Glutamate Luria salts (MG/L) medium (58), or Induction medium containing 1% glucose (61) at 28°C. Induction medium was used for attempts to reconstitute wild type *vir* gene induction, while MG/L and LB medium

were utilized for strains containing *virG*^{con} (i.e. pSY215 and pLG2). When appropriate, media was supplemented with ampicillin (100 μg/ml), gentamycin (20 μg/ml), kanamycin (50 μg/ml) and tetracycline (20 μg/ml) for *E. coli* and carbenicillin (100 μg/ml), gentamycin (100 μg/ml), kanamycin (100 μg/ml) and tetracycline (5 μg/ml) for *A. tumefaciens*. For determinations of β-galactosidase activity, 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside (X-Gal) and isopropylthio-β-galactoside (IPIG) were included at a final concentration of 75 μg/ml and 1 mM, respectively. Acetosyringone (Sigma) and glucose was included, when necessary at 200 μM and 1%, respectively. For induction assays, *E. coli* stain MC4100 containing constructs indicated in Table 1 were grown to stationary phase in 5 ml of appropriate medium, containing antibiotics as required. These overnight cultures were used to inoculate 125 ml flasks containing 30 ml of identical medium using 0.5 ml as an inoculum. The cultures were incubated at 28°C with shaking for 16 hours and assayed for β-galactosidase activity according to the method of Miller (40). For reconstitution of virulence gene expression in *E. coil,* plasmid constructs pSY2O4, pLG2, pGP159 and pSL107 were introduced into MC4100 by electroporation. Construct pH098 containing *lac-driven A. turnefaciens rpoA* was then introduced into these strains and initially screened on LB medium (pSY2O4 and pLG2) or induction medium (pGP159 and pSLlO7) containing appropriate antibiotics, 1 mM IPTG, 75 mg/mi X-Gal and 200 μM acetosyringone. Induction assays for these strains were carried out as described above.

Isolation and subcloning of *rpoA* locus

A cosmid clone library of *A. tumefaciens* strain Al36, a derivative of strain C58 lacking the Ti plasmid, in pVK102 was described previously (8). The cosmid clones were transformed into *E. coli* strain MC4100 containing plasmid pSY215. Transformants were initially screened for the development of a blue color on induction media containing X-Gal, indicating expression of the *virBp::lacZ* fusion. A cosmid clone, designated pBK2, was isolated by this procedure and was used for subsequent subcloning attempts, as outlined in Figure 1.

DNA sequencing was performed by PCR mediated Taq DyeDeoxy Terminator Cycle sequence on an Applied Biosystems model 377 DNA sequencer. The GCG sequence analysis software package (Genetics Computer Group, Madison, WI) and the BLAST software package (2) were used for all DNA and protein sequence analysis. The complete

nucleotide sequence of the 1.3 Kb PstI-StuI DNA fragment of pPS1.3 was determined on both strands. The *rpoA* sequence has been deposited into GenBank (accession # AF111855)

Overexpression and purification of proteins

For overproduction of the RpoA proteins, *rpoA* genes from *E. coli* and *A. tumefaciens* were PCR amplified from the MC4100 chromosome and plasmid pBX4.1 (Figure 1), respectively, using primers designed to contain a *BamHI* restriction site. [*E. colt* l)5'-CCA AAG AGA GGA TCC AAT GCA GGG-3', 2) 5'-CCT TAA CCT GGG ATC CGG TTA CTC G-3'; *A. tumefaciens* 1) 5'-GGA AGG ATC CAA GAT GAT TCA GAA GA-3', 2) 5'-CCT GGAA TCC TGC AGA TGA CTT ATC TG-3']. The PCR products were initially cloned into PCR2.1TOPO (Invitrogen, Inc.), and then subcloned into pQE vectors (Qiagen) to generate pECH4 and pZL-2, containing N-terminal His-tagged fusions to RpoA of *E. colt* and *A. tumefaciens,* respectively.

The His-tagged RpoA proteins of *A. tumefaciens* and *E. coli* were prepared from *E. coli* strain SG13009 (pREP4) containing pZL-2 or pECH4, respectively. Cells were grown at 37°C in 500 ml of L-Broth containing selective antibiotics to an OD_{60Q} of 0.3, and then induced with 1 mM IPTG for 3 hours at 37°C. Cells were harvested, resuspended in 20 ml of binding buffer (20 mM Tris-HCl pH7.9, 500 mM NaCl, *5%* Glycerol) and lysed by sonication. The lysates were cleared by low speed centrifugation and loaded on a Fast Flow Chelating Sepharose Column charged with Ni^{2+} (Pharmacia, Inc.). The column was attached to a Waters 650 Advanced Protein Purification System, washed extensively with loading buffer containing 25 mM imidazole, and bound protein eluted with buffer containing 100 mM imidazole. Fractions containing greater than 90% RpoA were precipitated by addition of ammonium sulfate to 75% saturation. The resulting pellet was dissolved in a storage buffer (20 mM Tris pH7.9, 40 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 50% Glycerol) and stored at 20°C.

Recombinant β β' and σ^{70} subunits of *E. coli* RNAP were each purified from overexpression strains as described previously (16, 51). Induction was initiated with 1 mM IPTG at 37°C for *3* hours and the proteins present in inclusion bodies were then purified according to Tang (55). The constitutively active VirG protein ($VirG^{con}$) was purified from *E.* coli as described previously (30).

RNAP holoenzymes were reconstituted from individually purified *E. coli* β β' and σ^{70}, and either *E. coli* or *A. tumefaciens* RpoA as described (3). The molar ration of α, β

and β' in the reconstitution reactions was 1:4:8. After reconstitution and thermoactivation in the presence of σ^{70}, RNAP preparations were further purified by gel exclusion chromatography on a Superose-6 column (Pharmacia) and ion exchange chromatography on a Resource Q column (Pharmacia). The purified RNAP holoenzymes were concentrated by filtration through a C-100 concentrator (Amicon, Inc.) to ~1 mg/ml, and stored in 50% glycerol storage buffer at -20°C.

In *Vitro* Transcription Assays

For analysis of abortive initiation, 2 pmol of recombinant RNAP holoenzyme containing either *E. coli* or *A. tumefaciens* RpoA was incubated with 10 pmol of template DNA in 10 μl of transcription buffer (40 mM Tris-HCl pH7.9, 40 mM KCl, 10 mM $MgCl_2$) for 10 minutes at 23°C in the presence or absence of the $VirG^{con}$ protein (2 pmol). As templates, a 130 bp EconRI-HindIII fragment of plasmid pAA121 containing the ga/Pl promoter (-63 to +44) or a 380 bp PCR product of plasmid pSM243cd (Stachel 1985) containing the virB promoter (-248 to +81) were used (4, 10). Abortive initiation reactions were initiated by addition of 0.5 mM initiating dinucleotide (CpA for ga/Pl promoter and ApU for virB promoter) and 0.5 μM [α-^{33}p] UTP (ga/Pl) or [α-^{33}p] ATP (virB) (3000Ci/mmol). After 25 incubation at 37°C, reactions were terminated by the addition of an equal volume of urea loading buffer. The reaction products were resolved on urea-PAGE (20% polyacrylamide, 19:1 acrylamide:bis) and visualized by autoradiography.

Mobility shift assays

Electrophoretic mobility shift DNA binding assays were carried out using a PCR amplified *virB* promoter labeled with (α-^{32}P] dCTP. PCR reactions included 20 μCi (α-^{32}P]dCTP, plasmid pSM243cd as template, Primer 1:5'-TTC CAC GGT GAC GCA TCG AAT G-3', and Primer 2:5'-CCC CGA TCT CTT AAA CAT ACC TTA TCT CC-3'. Unincorporated nucleotides were removed using the Wizard™ PCR Preps DNA Purification System kit from Promega. Mobility shift reaction mixtures contained 1600 cpm ^{32}P-labeled *virB* promoter, 50 mM KCl, 20 mM Tris-HCl pH 7.0, 10 mM $MgCl_2$, 1 mM DTT, 10% Glycerol and 20 μg/ml Herring sperm DNA. Where indicated, 1.5 μM VirG was added, which was sufficient to saturate all VirG binding sites on the promoter, and His-RpoA was added at 150-500 nM final concentration. Reactions were incubated for 30 minutes at 22°C, loaded onto 6% polyacrtlamide, 10% glycerol vertical slab gels in

0.5X TBE buffer (46) and electrophoresed at 20V/cm at 4°C for 2 hours. Following electrophoresis the gels were dried and autoradiographed overnight at room temperature.

Identification of an *A. tumefaciens* gene that is required for *vir* gene expression in *E. coli*

To reconstitute *vir* gene induction in *E. coli*, plasmid construct pGP159 containing *virA* and *virG* under the control of their native promoters and a *lacZ* reporter gene fused downstream of the *virB* promoter *(virBp::lacZ* fusion) was introduced into *E. coli* strain MC4100. The resulting strain failed to activate transcription of the fusion in response to acetosyringone *(AS)*. The inability of pGP159 to activate transcription may have been due to a lack of expression of the *virA/virG* genes, which are under the control of their native promoters. To address this possibility, the plasmid pSL107 was utilized, which contains *virA* and *virG* under the control of the *lac* promoter and *virBp:lacZ*. Introduction of pSL107 into MC4100 also failed to activate expression of the *vir* gene fusion in the presence of AS and IPTG. The observed lack of expression was not due to a lack at expression of *virA* and/or *virG* as both proteins were present at detectable levels by Western blot (data not shown). Since *VirA* and *VirG* were present, the *VirA/VirG* signal transduction mechanism may not be functional in *E. coli*. This possibility was addressed through the use of pSY215 which contains a constitutively active $virG^{con}$ under the control of the *lac* promoter and *vtrBp::lacZ*. The $virG^{con}$ is able to activate expression of the *virBp::lacZ* fusion *in A. tumefaciens* strains independent of *virA* and acetosyringone (17, 31, 45). When pSY215 was introduced into MC4100, once again no expression of the *virBp::lacZ* fusion was observed. This suggested that additional genes from *A. tumefaciens* may be required or that *E. coli* may contain specific repressors of *vir* gene expression.

To determine if additional genes from *A. tumefaciens* were required, a cosmid library constructed from chromosomal DNA of a Ti plasmidless strain A136, was introduced into MC4100(pSY215). Screening of the resulting transformants revealed the presence of a clone which produced a light blue color on colonies grown on induction media containing X-Gal and IPTG. This cosmid clone, designated pBK2, contains a 25 Kb DNA insert. In order to identify the gene residing in pBK2 required for expression of *virBp::lacZ*, overlapping subclones of pBK2 were generated which were introduced into MC4100(pSY215). Expression of the fusion was detected following introduction of pBX4.1, but not pBKS7.0 or pBKE4.8 (Fig. 1 & Table 2). The inability of pBKS7.0 and pBKE4.8 to activate expression of the fusion indicated that the 0.55 Kb region between

these two subclones is required for expression of the fusion. Given this information, a 1.3 Kb StuI-PstI DNA fragment from pBX4.1 was isolated and subcloned it into pTZ18R and pTZ19R yielding pPS1.3R and. PPSI.3, respectively. This resulted in two constructs in which the *lac* promoter on the vector drives transcription from either end of the fragment. Expression of the *vir* fusion was detected only with pPS1.3, indicating the absence of promoter elements in the fragment and the responsible gene is in the direction of *PstI* to SruI. Subclone pPS1.3 resulted in the highest level of expression of the fusion, almost 15 fold higher than pBK2, and 40 fold higher than the vector control. This could be due to both increased copy number of the gene and increased gene expression by the strong *lac* promoter (Table 2).

Characterization of the identified gene

The DNA sequence of the entire 1.3 Kb *StuI-PstI* fragment was obtained from both strands. Analysis of the DNA sequence revealed an open reading frame of 1,008 base pairs, in the predicted transcriptional direction, sufficient to encode a polypeptide of 336 amino acids as shown in Figure 2A. Nucleotide and protein searches of GenBlank and Swiss-Prot databases indicated a high degree of similarity to *rpoA* genes, encoding the α subunit of RNAP. Comparison with *rpoA* from *E. coli* indicated 62.15% sequence similarity and 51.4% sequence identity at the amino acid level as shown in Figure 3. Three highly conserved regions can be identified from the distribution of homologous amino acid residues between *E. coli* and *A. tumefaciens rpoA* homologues. One region extends from residue 30 to 51 near the N-terminus (20 of 22 identical), with the other two present in the C-terminal domain extending from residue 256 to 270 (13 of 15 identical) and from residue 276 to 315 (30 of 42 identical). A notable difference is the presence of an additional eight residues at the C-terminus of the *A. tumefaciens* RpoA, compared to RpoA of *E. coli*. A potential Shine-Dalgamo sequence, GAAGGT, was found extending from -7 to -12 bp upstream of the proposed ATG initiation codon of *rpoA*. Analysis of partial DNA sequence obtained from pBKS7.0 and pBKE4.8 indicated the presence of regions upstream and downstream of *rpoA* with a high degree of sequence similarity to *rpsK* and *rplQ*, encoding S11 and L17 ribosomal proteins, respectively, possibly forming an operon structure similar to *E. coli*.

Confirmation of the ORF was achieved by construction of a His-tagged *rpoA* fusion as described above, which yielded a polypeptide of the predicted size (~ 37 Kd) that is

slightly larger than *E. coli* RpoA (Fig. 4A). Furthermore, the DNA clone from *A. tumefaciens* was able to complement a temperature sensitive *rpoA* mutant of *E. coli*, HN317, proving that it does encode a functional homologue of the RNAP α subunit.

In vitro transcription of the *virB* promoter

In vitro assays involving the *rpoA* subclones and a constitutive $virG^{con}$, *in vitro* transcription assays were performed. Purified individual components of *E. coli* RNAP, β β' and σ^{70}, were mixed with either His-RpoA of *E.coli* or *A. tumefaciens* and high molecular weight RNAP complexes were purified by sizing column (see Materials and Methods). As shown in Figure 4A, both RpoA molecules were able to successfully assemble with the *E. coli* β β' and σ^{70} subunits into complete RNAP holoenzymes. When tested for *in vitro* transcription, both RNAP holoenzymes were equally efficient in initiating transcription from a α^{70} dependent *E. coli ga/P1* promoter (Fig. 4B), demonstrating that the hybrid RNAP containing *RpoA* (α-subunit of RNAP) of *A. tumefaciens* is a functional enzyme. Furthermore, no significant differences were detected in the amount of the transcript produced in the presence or absence *of* $virG^{con}$. When the *virB* promoter was used as a template, the *E. coli* KNAP could activate low level transcription but no difference was evident with or without the $VirG^{con}$ protein. In contrast, the hybrid RNAP containing *A. tumefaciens* RpoA was able to activate transcription from the *virB* promoter at low levels, and addition of the $VirG^{con}$ increased transcription by 4-5 fold as measured by quantification of the gel (Fig. 4B). These results confirm the *in* vivo assays demonstrating that only RNAP containing RpoA of *A. tumefaiens* is able to efficiently initiate transcription from the *virB* gene promoter in a $VirG^{con}$ dependent manner. The inability of *E. coli* RNAP to efficiently express the *vir* fusion even in the presence of the $VirG^{con}$ suggests that *E. coli* RpoA can not make the required contacts with the $VirG^{con}$ protein.

Specific interaction between the $VirG^{con}$ and RpoA of *A. tumefaciens*

Since the C-terminal domain of the *E. coli* RpoA was known to interact with the A+T rich "UP element of certain promoters (Ross 1993), the *E. coli* and *A. tumefaciens* RpoA proteins were tested to determine if these proteins have different affinities for the *virB* promoter. As shown in Figure 5 (lanes 3, 4 and *5), A. tumefaciens* RpoA was able to shift the mobility of the labeled *virB* promoter at the highest concentration used (600nM), whereas the same concentration of *E. coli* RpoA did not, suggesting that the RpoA of *A. tumefaciens* has a higher affinity for the *virB* promoter than RpoA of *E. coli*. Similar

mobility shift assays were carried out to determine if His-RpoA of E. coli and A tumefaciens interact differently with VirG^{con} at the *virB* promoter. Increasing amounts of RpoA were used in. combination with a saturating quantity of VirG^{con} for the *virB* promoter (Fig. 5, lane 2) The concentration of VirG^{con} used was determined through separate mobility shift assays in which increasing amounts of VirG^{con} resulted in two separate shifts in mobility, corresponding to binding of VirG^{con} at the two *vir* boxes of the *virB* promoter (data not shown). At the three concentrations of *E. coli* RpoA used, there was no additional shift in the mobility of the promoter VirG^{con} complex (Fig. 5A, lanes 6. 7 and 8). However, when RpoA from *A. tumefaciens* was used, two separate shifts were observed (Fig 5B, lanes 6, 7 and 8) which suggests specific interactions between VirG^{con} and RpoA from *A. tumefaciens*. These results provide further evidence suggesting that RpoA from *A. tumefaciens* may possess a higher affinity for VirG^{con} compared to RpoA from *E. coli*.

Combination of *virA/G* and *rpoA of A.* tumefaciens is insufficient to reconstitute acetosyringone mediated *vir* gene induction in E. coli

To determine if the signaling mechanism, resulting in *vlr* gene activation, can be reconstituted in *E. coli*, a wild type *virA* and *virG* was used in combination with *A. tumefaciens rpoA*. M04100 harboring two plasmid constructs, one containing a *virBp::lacZ* fusion as well as wild type *virA/virG* under their native promoters (pGP159), and. the other containing a *lac*-driven *A. tumefaciens rpoA* gene (pH098), did not show *any* significant increase in β-galactosidase activity in the presence of acetosyringone (data not shown). The possibility that lack of expression of *virA* and/or *virG* may account for this result was again addressed through the use of pSL107 which contains *lac-driven virA/virG* and *virBp::lacZ*. However, it was not possible to obtain significant expression of the fusion in MC4100 harboring pSL107 and pH098. Introduction of pH098 into MC4100(pLG2), containing *lac-driven VirG^{con}* and *virBp::lacZ,* however, resulted in a significant increase in β-galactosidase activity, demonstrating that pH098 is able to produce a functional RpoA protein. These results suggest that the signal transduction mechanism of VirA/VirG may not be functional in *E. coli*.

The present invention discloses that it is possible to obtain the expression of heterologous genes that previously could not have been expressed in a host, such as *E. coli*, by including a *rpoA* gene or a portion thereof from the same host genus as the expressed gene is obtained The ability to use *E. coli* as a heterologous system provides investigators

with a valuable tool for studying these various processes. The identification of a chromosomally encoded *A. tumefaciens rpoA* gene and the demonstration that it constitutes one of the components required for expression of a *virBp::lacZ* gene fusion in a heterologous *E. coli* background supports the present invention. The *rpoA* gene of *E. coli* has been extensively studied, particularly with regard to interactions with transcriptional regulators, and suggests that interaction between VirG and RpoA may be required for efficient transcription of virulence genes.

The inability of pGP159 or pSL107 to activate transcription of the *virBp::lacZ* fusion suggested that either signal transduction between VirA and VirG was not functional, or that additional gene(s) were required from *A. tumefaciens* for activation. The use of pSY215 containing a virG^{con} mutant allowed us to evaluate *vir* gene expression in a *virA*-independent manner, eliminating the need for signal transduction. The lack of expression obtained with pSY215 in *E. coli,* combined with its ability to function in a Ti plasmidless *A. tumefaciens* strain suggested that additional *A. tumefaciens* gene(s) were required for expression. The introduction of pPS1.3 containing *lac* driven rpoA into MC4100(pST215) resulted in a significant increase (40 fold) in transcription of the *virBp::lacZ* fusion compared to the control vector. Verification that *rpoA* is required was obtained through the use of subclone pPS1.3R, which did not activate expression of the fusion. This construct is identical to pPS1.3, but the direction of transcription of rpoA is opposite to that of the *lac* promoter. The observation that *A. tumefaciens rpoA* was able to complement a temperature sensitive rpoA mutant in *E. coli* demonstrates an ability to function at essential *E. coli* promoters. This is evident from the in vitro transcription assay where the hybrid NAP was equally effective as *E. coli* RNAP in transcribing a σ^{70}-dependent *ga/P1* promoter (Fig. 4B). While the expression of the *virBp::lacZ* fusion (Table 1) was significantly increased, the level of expression was relatively low in comparison to expression *in A. tumefaciens* (31). The relatively low expression of the *virBp::lacZ* fusion may have been a consequence of the presence of RNAP containing RpoA of *E. coli*. In order to remove possible interference from *E. coli* RpoA, *in vitro* transcription assays using reconstituted RNAP holoenzymes containing His-RpoA from either *E. coli* or *A. tumefaciens* were carried out. Using purified *E. coli* β, β' and α^{70} subunits, it was demonstrated that both of the His-RpoA were able to assemble into multi-subunit RNAP holoenzymes (Fig. 4A). The results of the *in vitro* transcription assays demonstrated that VirG^{con}-dependent transcription of the

virBp::lacZ fusion requires RNAP containing *A. tumefaciens* RpoA, although the two reconstituted holoenzymes exhibited essentially identical activity in transcription from the ga/P1 promoter, with no significant difference in the presence or absence of $VirG^{con}$ (Fig 4B). Another possible explanation for the relatively low induction in *E. coli* may due to the presence of *E. coli* sigma factors in the RNAP holoenzymes. It is conceivable that *E. coli* sigma factors have a lower affinity for the virB promoter than sigma factors from *A. turnefaciens*. Although the vegetative sigma factor from *A. turnefaciens* has been identified (50), it *is* unclear whether this or an alternative sigma factor is involved in *vir* gene transcription.

Previous reports have identified the presence of an "UP element" in certain *E. coli* promoters which is required for optimal transcription (14, 41, 47). This element extends from -40 to -60 bp upstream of the transcription start site and is highly A+T rich. Interestingly, the *virB* promoter contains an A+T rich sequence from -40 to -60 that overlaps wit the VirG binding sites. Whether this region of the promoter constitutes a true "UP element" is unknown. From the gel shift assays, the *A. tumefaciens* RpoA appears to have a higher affinity for the *virB* promoter than *E. coli* RpoA (Fig 5A and B), although the importance of this observation is unclear at this time. The results of the mobility shift assay suggest that *E. coil* RpoA is unable to bind to $VirG^{con}$ at the *virB* promoter. In contrasts, *A. tumefaciens* RpoA appears to exhibit cooperative binding with two distinct shifts in the mobility of the $VirG^{con}$-*virB* promoter complex. Taken together, these results indicate that RNAP containing *E. coli* RpoA may be unable to interact effectively with $VirG^{con}$, and therefore can not activate transcription from the *virB* promoter. Since the *virB* promoter contains two binding sites for VirG, the presence of two shifts obtained with increasing amounts of *A. tumefaciens* RpoA may be a consequence of RpoA interacting with VirG at each *vir* box.

The two component regulatory system, composed of *vir*A and *vir*G, is indispensible for transcription of virulence genes within *A. tumefaciens*. However, *vir*A and *vir*G are insufficient to activate transcrition from virulence gene promoters within *E. coli* cells, indicating a requirement for additional *A. tumefaciens* gene(s).

In examining *vir* gene expression in *E. coli,* attempts to reconstitute wild type virulence gene expression in *E. coli* were not successful. The use of *virA* and *virG* under the control of the *lac* promoter means that sufficient levels of *vir*A and *vir*G should be

present for signal transduction to take place. One possible explanation may be that *E. coli* is unable *to* correctly insert *vir*A into the inner membrane. Alternatively, even, though *vir*A may be inserted into the inner membrane correctly, dimerization of *vir*A which is required for activity in. *A. turnefuciens* (43) may be defective. A more likely explanation may be that additional gene(s) from *A. tumefaciens* are required for efficient signal transduction. An unresolved question is the exact mechanism of sensing of phenolic inducers by the *vir*A/*vir*G system. The two possible mechanisms involve direct binding of the inducer by *vir*A, or binding by a second receptor which then interacts with *vir*A. Although genetic evidence supporting direct binding of inducers by *vir*A has been reported (36, 37), all attempts to demonstrate direct binding by *vir*A have been unsuccessful. Conversely, there have been reports in which binding of phenolic compounds by proteins other than *vir*A have been detected (13, 35), although there is no evidence to link these proteins with *vir* gene induction. The search for additional *A. tumefaciens* gene(s) involved in the signal transduction should be simplified by determining that *vir*G^{con} mediated expression of virulence genes requires RpoA from *A. tumefaciens*. The present invention provides the basis to examine *vir* gene expression as well as the T-DNA transfer process in *E. coli*.

This specific example shows that for the expression of *vir* genes in *E. coli*, both *rpoA* and *virG* from *A. tumefaciens* are required for transcriptional activation of a *vir* promoter in *E. coli*. It has been determined that the *rpoA* gene, encoding the α subunit of RNAP, confers significant expression of a virBp;;lacZ fusion in *E.coli* in the presence of an active transcriptional regulator *vir*G gene. *In vitro* transcription assays were conducted using either reconstituted *E. coli* RNAP or hybrid RNAP in which the α subunit was derived from *A. tumefaciens*. Both RNAPs were equally efficient in transcription from a σ^{70}- dependent *E. coli* galP1 promoter, however, only the hybrid RNAP was able to transcribe the *vir*B promoter in a *vir*G-dependent manner.

Evidence is also presented which indicates that *vir*G interacts with rpoA from *A. tumefaciens* but not with rpoA from *E. coli*. This observation suggests that in order for successful transcription of *vir* genes to occur, specific interaction(s) between the *A. tumefaciens* α subunit of RNAP and *vir*G is required.

As shown in Figure 3, one of the major difference between the RpoA sequences of *A. tumefaciens* and *E.coli* is the presence of an extra 8 consecutive amino acids at the C-

terminus of *A. tumefaciens* RpoA (amino acids 329 to 336). To test if these C-terminal 8 consecutive amino acids are important in mediating *vir* gene activation in *E.coli*, a plasmid construct was generated from pPS1.3 by deleting the DNA coding for the last 8 amino acids using site directed mutagenesis. The resulting plasmid pAD8 encodes 328 amino acid long *A. tumefaciens* RpoA, lacking the original amino acids from 329 to 336.

The *vir* gene activating ability of this construct was tested by introducing pAD8 into *E.coli* strain MC4100 harboring pSY215 which contains *virA*, *virG* and *virB::lacZ* fusion. As negative and positive controls, vector plasmid pQE31 or pPS1.3 which encodes intact RpoA of *A. tumefaciens* was introduced into MC4100(pSY215), respectively. The *vir* gene activation was monitored by measuring the β-galactosidase activities. As shown in Table 3, pAD8 failed to activate β-galactosidase activity whereas the pPS1.3 induced a high level β-galactosidase activity. These results indicate that the C-terminal 8 amino acids of *A. tumefaciens* RpoA are essential for the *vir* gene activation in *E.coli*.

These experiments also demonstrate that incorporation of a *rpoA* gene of a prokaryote, such as *A.tumefaciens*, into an expression vector will promote the expression of genes from the prokaryotic cells in other prokaryotic cells, such as *E. coli*. Expression of this expression vector comprising at least a portion of an *A. tumefaciens* gene is a useful gene delivery system in heterologous hosts for gene delivery and gene therapy. Further, incorporation of at least a portion of an *rpoA* gene derived from the same cells as one or more heterologous genes, into a vector containing the heterologous genes, provide improved systems for expressing the heterologous genes. These expression vectors which comprise one or more heterologous genes and at least a portion of an *rpoA* gene derived from the same cells as the heterologous genes, are useful in the production of selected metabolites, by expression genes for an entire metabolic pathway in a heterologous host.

The following list of publications referred to in the specification are herein incorporated in their entirety by reference.

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Table 1. Bacterial Strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Source
E. coli		
DH5α	*recA endAI hsdR17 supE4 gyrA96 reLAI (lacZYA-argF)U169 (80dlacZ M15)*	(56)
MC4100	F` araD139 (argF-kac)U169 rpsL150 relA fib5301 ptsF25deoC1	(57)
HN317	rpoA112^{ts}St^{t}	(58)
Plasmids		
pGP159	*virA. virG & virBp*::lacZ; IncP, Ap^{t},TC^{t}	(59)
pSM234acd	*virA & virBp*::lacZ: IncP. Km^{r}	(45)
pLG2	*lacp*::*virG*(N54D) & *virBp*::lacZ; IncP, Ap^{r}Tc^{t}	This study
pSL107	*lacp*::*virG* & *vir*Bp::*lacZ*:IncW,AP^{r}Tc^{t}	This study
pPC401(N54D)	*lacp*::*virG*(N54D) in pTZ18R.Ap^{t}	This study
pSY215	*virBp*::lacZ & *lacp*::virG(N54D); IncW, Gm^{r}	This study
pVK102	Cosmid cloning vector; IncP, Km^{t},Tc^{r}	(60)
pTZ18R/pTZ19R	Cloning vector; ColEl.Ap^{r}	USB
pBK2	pVK102 with 25 Kb chromosomal insert from A.*tumefaciens strain* A136, Tc^{t}	This Study
pBKS2-2	pTZ18R with 7.1 Kb *Sau*3A insert from pBK2	This study
pBX4.1	pTZ18R with 4.3 Kb *Bam*HI-XbaI insert from pBKS2-2	This study
pBKS7.0	pTZ18R with 4.2 Kb *Sau*3A-Sa*l*I insert from pBKS2-2	This study
pBKE4.8	pTZ18R with 1.7 Kb *Eco*RI-*Sau*3A insert from pBKS2-2	This study
pPS1.3R	pTZ18R with 1.3 Kb *PstI-Stu*I insert from pBKS2-2	This study
pPS1.3	pTZ19R with 1.3 Kb *Pst*I-*Stu*I insert from pBKS2-2	This study
pZL-2	Overproducer of *A. tumefaciens* His-RpoA: Ap^{t}	This study
pECH4	Overproducer of *E. coli* His-RpoA.Ap^{t}	This study
pH098	*lacp*::rpoA of *A. tumefaciens* from pPS1.3: IncW.Km^{t}	This study

Table 2. RpoA mediated expression of *vir*BP::lacZ fusion in *E. coli* MC4100 containing *virG*^{test}

Constructs	LB medium		MG/L medium	
	β-Galactosidase[b]	Fold Activation[c]	β-Galactosidase[b]	Fold Activation
pBK102	2.46	NA	2.35	NA
pBK2	5.2	2.11 X	6.56	2.79 X
pTZ18R	2.24	NA	2.51	NA
pBKS2-2	13.67	6.1 X	7.25	2.89 X
pBX4.1	26.42	11.8 X	8.11	3.23 X
pBKS7.0	1.68	0.75 X	2.46	0.98 X
pBKE4.8	2.36	1.05 X	1.63	0.65 X
pBP3.0	5.45	2.43 X	2.83	1.13 X
pPS1.3R	2.13	0.95 X	1.71	0.68 X
pPS1.3	76.14	40.0 X	37.69	15.0 X

The *virBp*::*lacZ* expression assays were carried out in LB and MG/L medium for 16 hours as described in the text, IPTG was added at a final concentration of 1 mM. *E. coli* MC4100 containing pSY215 (*virG*^{con} + *virBp*::lacZ).[b] Values are the average of three replicates.

[c] Values indicate an increase or decrease in activity compared to the appropriate control vector. NA, Not Applicable.

Table 3. β-galactosidase activities

E.coli strain MC4100(pSY215) plus plasmid shown in right	pPS1.3	pAD8	pQE31
β-galactosidase in Miller Units*	140 (+/-25)	4 (+/-1)	3 (+/-2)

* Miller Units = Defined in Ref. 68.

We claim:

1. An isolated nucleic acid molecule encoding at least a portion of the α subunit of an RNAP of *Agrobacterium*.

2. An isolated nucleic acid molecule encoding the complete α subunit of an RNAP of *Agrobacterium*.

3. An isolated nucleic acid molecule encoding the complete α subunit of an RNAP of *Agrobacterium*, wherein the amino acid sequence of said α subunit is depicted in Figure 2A.

4. An isolated nucleic acid molecule encoding at least a portion of the α subunit of an RNAP of *Agrobacterium*, wherein the amino acid sequence of said α subunit is depicted in Figure 2A.

5. The nucleic acid molecule of claim 4, wherein said portion of the amino acid sequence as depicted in Figure 2A comprises at least amino acid residues 157 to 336.

6. The nucleic acid molecule of claim 4, wherein said portion of the amino acid sequence as depicted in Figure 2A comprises less than amino acid residues 157 to 336.

7. The nucleic acid molecule of claim 4, wherein said portion of the amino acid sequence as depicted in Figure 2A comprises at least 8 consecutive amino acid residues in length.

8. The nucleic acid molecule of claim 7, wherein said portion of the amino acid sequence as depicted in Figure 2A comprises amino acid residues 329 to 336.

9. An isolated nucleic acid molecule comprising the nucleic acid sequence as depicted in Figure 2B.

10. An isolated nucleic acid molecule comprising at least a portion of the nucleic acid sequence as depicted in Figure 2B.

11. A hybrid nucleic acid molecule for the expression of at least one heterologous gene in a host cell, wherein said hybrid nucleic acid molecule comprises a first nucleic acid sequence encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the host cell and a second nucleic acid sequence encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the heterologous gene.

12. The isolated nucleic acid molecule of claim 11, wherein said second nucleic acid sequence is obtained from a prokaryotic cell selected from the group consisting of

Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

13 The isolated nucleic acid molecule of claim 12, wherein said second nucleic acid sequence is obtained from the genus *Agrobacterium*, *Mycobacterium*, *Chlamydia*, *Pseudomonas* or *Streptomyces*.

14. The nucleic acid molecule of claim 13, wherein said second nucleic acid sequence is obtained from *Agrobacterium*.

15. The nucleic acid molecule of claim 14, wherein said second nucleic acid sequence encodes the amino acid sequence as depicted in Figure 2A.

16. The nucleic acid molecule of claim 14, wherein said second nucleic acid sequence encodes at least a portion of the amino acid sequence as depicted in Figure 2A.

17. The nucleic acid molecule of claim 16, wherein said portion of the amino acid sequence as depicted in Figure 2A comprises at least amino acid residues 157 to 336.

18. The nucleic acid molecule of claim 16, wherein said portion of the amino acid sequence as depicted in Figure 2A comprises less than amino acid residues 157 to 336.

19. The nucleic acid molecule of claim 16, wherein said portion of the amino acid sequence as depicted in Figure 2A comprises at least 8 consecutive amino acid residues in length.

20. The nucleic acid molecule of claim 19, wherein said portion of the amino acid sequence as depicted in Figure 2A comprises amino acid residues 329 to 336.

21. The hybrid nucleic acid molecule of claim 11, wherein said first nucleic acid sequence is obtained from a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

22. The nucleic acid molecule of claim 21, wherein said first nucleic acid sequence is obtained from the genus *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*.

23. A nucleic acid construct comprising a nucleic acid molecule of any one of claims 1-22 operably linked to a promoter.

24. A vector comprising the nucleic acid construct of claim 23.

25. A host cell comprising a nucleic acid construct of claim 23 or a vector of claim 24, wherein said promoter is operable in said host cell.

26. A host cell of claim 25, further comprising at least one heterolgous gene operably linked to a promoter.

27. A host cell of claim 26, wherein said heterolgous gene and promoter are contained in a vector.

28. A host cell of any one of claims 25-27, wherein said host cell is a prokaryotic cell or a eukaryotice cell.

29. A host cell of claim 28, wherein said host cell is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

30. A host cell of claim 29, wherein said host cell is an *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*.

31. A method of expressing at least one heterologous gene in a host cell comprising:

transforming said host cell with a nucleic acid molecule of 23 and with at least one heterologous gene operably linked to a promoter; and

culturing said transformed host cell under conditions wherein at least one of said heterologous genes is expressed in said host cell.

32. The method of claim 31, further comprising transforming said host cell with at least one gene encoding a heterologous transcriptional regulator obtained from the same genus as the source of the heterologous gene.

33. The method of claim 32, wherein said transcirptional regulator is a transcriptional activator that interacts with the α subunit of the RNAP to enhance the expression of said heterologous gene.

34. The method of any one of claims 31 - 33, wherein said heterologous gene comprises multiple genes or operons in the same metabolic pathway.

35. The method of claim 31, wherein said nucleic acid molecule and said at least one heterologous gene are contained in at least one vector.

36. The method of claim 31, wherein said host cell is transformed with a vector comprising the nucleic acid molecule and with at least one vector comprising at least one heterologous gene.

37. The method of claim 31, wherein said host cell is a prokaryotic cell or a eukaryotic cell.

38. The method of claim 37, wherein said host cell is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fact bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

39. The method of claim 38, wherein said host cell is an *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*.

40. The method of claim 39, wherein said prokaryotic cell is an *Escherichia*.

41. The method of claim 31, wherein said genus of the source of the heterolgous gene is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

42. The method of claim 41, wherein said genus is *Agrobacterium*, *Mycobacterium*, *Chlamydia*, *Pseudomonas* or *Streptomyces*.

43. The method of claim 42, wherein said genus is obtained from *Agrobacterium*.

44. A method of expressing at least one heterologous gene in a host cell comprising:

transforming said host cell with a nucleic acid molecule encoding a complete α subunit of an RNA polymerase obtained from the same genus as the heterologous gene or a portion of said α subunit of the RNA polymerase operably linked to a promoter, and with at least one heterologous gene operably linked to a promoter; and

culturing said transformed host cell under conditions wherein at least one of said heterologous genes is expressed in said host cell.

45. The method of claim 44, further comprising transforming said host cell with at least one gene encoding a heterologous transcriptional regulator obtained from the same genus as the source of the heterologous gene.

46. The method of claim 45, wherein said transcirptional regulator is a transcriptional activator that interacts with said α subunit of the RNAP to enhance the expression of said heterologous gene.

47. The method of any one of claims 44-46, wherein said heterologous gene comprises multiple genes or operons in the same metabolic pathway.

48. The method of claim 44, wherein said nucleic acid molecule and said at least one heterologous gene are contained in at least one vector.

49. The method of claim 44, wherein said host cell is transformed with a vector comprising the nucleic acid molecule and with at least one vector comprising at least one heterologous gene.

50. The method of claim 44, wherein said host cell is a prokaryotic cell or a eukaryotic cell.

51. The method of claim 50, wherein said host cell is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, aicd-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

52. The method of claim 51, wherein said host cell is from the genus *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*.

53. The method of claim 52, wherein said prokaryotic cell is an *Escherichia*.

54. The method of claim 44, wherein said genus of the source of the heterolgous gene is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

55. The method of claim 54, wherein said genus is *Agrobacterium*, *Mycobacterium*, *Chlamydia*, *Pseudomonas* or *Streptomyces*.

56. A purified α subunit of an RNA polymerase of *Agrobacterium* or a portion thereof.

57. A purified α subunit of an RNA polymerase of *Agrobacterium* of claim 55 or a portion thereof wherein the amino acid sequence of said α subunit is depicted in Figure 2A or a portion thereof.

58. A purified α subunit of an RNA polymerase of *Agrobacterium* of claim 55 or portion thereof, wherein the α subunit of RNA polymerase is encoded by the nucleic acid is depicted in Figure 2B or a portion thereof.

AMENDED CLAIMS
[received by the International Bureau on 15 August 2000 (15.08.00);
original claims 3-10, 15-20, 28, 34, 57 and 58 amended;
remaining claims unchanged (6 pages)]

1. An isolated nucleic acid molecule encoding at least a portion of the α subunit of an RNAP of *Agrobacterium*.

2. An isolated nucleic acid molecule encoding the complete α subunit of an RNAP of *Agrobacterium*.

3. An isolated nucleic acid molecule encoding the complete α subunit of an RNAP of *Agrobacterium*, wherein the amino acid sequence of said α subunit is depicted in Figure 2A (SEQ ID NO:1).

4. An isolated nucleic acid molecule encoding at least a portion of the α subunit of an RNAP of *Agrobacterium*, wherein the amino acid sequence of said α subunit is depicted in Figure 2A (SEQ ID NO:1).

5. The nucleic acid molecule of claim 4, wherein said portion of the amino acid sequence as depicted in Figure 2A (SEQ ID NO:1) comprises at least amino acid residues 157 to 336.

6. The nucleic acid molecule of claim 4, wherein said portion of the amino acid sequence as depicted in Figure 2A (SEQ ID NO:1) comprises less than amino acid residues 157 to 336.

7. The nucleic acid molecule of claim 4, wherein said portion of the amino acid sequence as depicted in Figure 2A (SEQ ID NO:1) comprises at least 8 consecutive amino acid residues in length.

8. The nucleic acid molecule of claim 7, wherein said portion of the amino acid sequence as depicted in Figure 2A (SEQ ID NO:1) comprises amino acid residues 329 to 336.

9. An isolated nucleic acid molecule comprising the nucleic acid sequence as depicted in Figure 2B (SEQ ID NO:2).

10. An isolated nucleic acid molecule comprising at least a portion of the nucleic acid sequence as depicted in Figure 2B (SEQ ID NO:2).

11. A hybrid nucleic acid molecule for the expression of at least one heterologous gene in a host cell, wherein said hybrid nucleic acid molecule comprises a first nucleic acid sequence encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the host cell and a second nucleic acid sequence encoding at least a

portion of the α subunit of an RNAP obtained from the same genus as the heterologous gene.

12. The isolated nucleic acid molecule of claim 11, wherein said second nucleic acid sequence is obtained from a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

13 The isolated nucleic acid molecule of claim 12, wherein said second nucleic acid sequence is obtained from the genus *Agrobacterium*, *Mycobacterium*, *Chlamydia*, *Pseudomonas* or *Streptomyces*.

14. The nucleic acid molecule of claim 13, wherein said second nucleic acid sequence is obtained from *Agrobacterium*.

15. The nucleic acid molecule of claim 14, wherein said second nucleic acid sequence encodes the amino acid sequence as depicted in Figure 2A (SEQ ID NO:1).

16. The nucleic acid molecule of claim 14, wherein said second nucleic acid sequence encodes at least a portion of the amino acid sequence as depicted in Figure 2A (SEQ ID NO:1).

17. The nucleic acid molecule of claim 16, wherein said portion of the amino acid sequence as depicted in Figure 2A (SEQ ID NO:1) comprises at least amino acid residues 157 to 336.

18. The nucleic acid molecule of claim 16, wherein said portion of the amino acid sequence as depicted in Figure 2A (SEQ ID NO:1) comprises less than amino acid residues 157 to 336.

19. The nucleic acid molecule of claim 16, wherein said portion of the amino acid sequence as depicted in Figure 2A (SEQ ID NO:1) comprises at least 8 consecutive amino acid residues in length.

20. The nucleic acid molecule of claim 19, wherein said portion of the amino acid sequence as depicted in Figure 2A (SEQ ID NO:1) comprises amino acid residues 329 to 336.

21. The hybrid nucleic acid molecule of claim 11, wherein said first nucleic acid sequence is obtained from a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

22. The nucleic acid molecule of claim 21, wherein said first nucleic acid sequence is obtained from the genus *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*.

23. A nucleic acid construct comprising a nucleic acid molecule of any one of claims 1-22 operably linked to a promoter.

24. A vector comprising the nucleic acid construct of claim 23.

25. A host cell comprising a nucleic acid construct of claim 23, wherein said promoter is operable in said host cell.

26. A host cell of claim 25, further comprising at least one heterologous gene operably linked to a promoter.

27. A host cell of claim 26, wherein said heterologous gene and promoter are contained in a vector.

28. A host cell of claim 26, wherein said host cell is a prokaryotic cell or a eukaryotic cell.

29. A host cell of claim 28, wherein said host cell is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

30. A host cell of claim 29, wherein said host cell is an *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*.

31. A method of expressing at least one heterologous gene in a host cell comprising:

transforming a host cell with a nucleic acid molecule of 23 and with at least one heterologous gene operably linked to a promoter; and

culturing said transformed host cell under conditions wherein at least one of said heterologous genes is expressed in said host cell.

32. The method of claim 31, further comprising transforming said host cell with at least one gene encoding a heterologous transcriptional regulator obtained from the same genus as the source of the heterologous gene.

33. The method of claim 32, wherein said transcriptional regulator is a transcriptional activator that interacts with the α subunit of the RNAP to enhance the expression of said heterologous gene.

34. The method of claim 31, wherein said heterologous gene comprises multiple genes or operons in the same metabolic pathway.

35. The method of claim 31, wherein said nucleic acid molecule and at least one of said heterologous genes are contained in at least one vector.

36. The method of claim 31, wherein said host cell is transformed with a vector comprising the nucleic acid molecule and with at least one vector comprising at least one heterologous gene.

37. The method of claim 31, wherein said host cell is a prokaryotic cell or a eukaryotic cell.

38. The method of claim 37, wherein said host cell is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fact bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

39. The method of claim 38, wherein said host cell is an *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*.

40. The method of claim 39, wherein said prokaryotic cell is an *Escherichia*.

41. The method of claim 31, wherein said genus of the source of the heterologous gene is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

42. The method of claim 41, wherein said genus is *Agrobacterium*, *Mycobacterium*, *Chlamydia*, *Pseudomonas* or *Streptomyces*.

43. The method of claim 42, wherein said genus is obtained from *Agrobacterium*.

44. A method of expressing at least one heterologous gene in a host cell comprising:

transforming said host cell with a nucleic acid molecule encoding a complete α subunit of an RNA polymerase obtained from the same genus as the heterologous gene or a portion of said α subunit of the RNA polymerase operably linked to a promoter, and with at least one heterologous gene operably linked to a promoter; and

culturing said transformed host cell under conditions wherein at least one of said heterologous genes is expressed in said host cell.

45. The method of claim 44, further comprising transforming said host cell with at least one gene encoding a heterologous transcriptional regulator obtained from the same genus as the source of the heterologous gene.

46. The method of claim 45, wherein said transcriptional regulator is a transcriptional activator that interacts with said α subunit of the RNAP to enhance the expression of said heterologous gene.

47. The method of any one of claims 44-46, wherein said heterologous gene comprises multiple genes or operons in the same metabolic pathway.

48. The method of claim 44, wherein said nucleic acid molecule and said at least one heterologous gene are contained in at least one vector.

49. The method of claim 44, wherein said host cell is transformed with a vector comprising the nucleic acid molecule and with at least one vector comprising at least one heterologous gene.

50. The method of claim 44, wherein said host cell is a prokaryotic cell or a eukaryotic cell.

51. The method of claim 50, wherein said host cell is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

52. The method of claim 51, wherein said host cell is from the genus *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*.

53. The method of claim 52, wherein said prokaryotic cell is an *Escherichia*.

54. The method of claim 44, wherein said genus of the source of the heterologous gene is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

55. The method of claim 54, wherein said genus is *Agrobacterium*, *Mycobacterium*, *Chlamydia*, *Pseudomonas* or *Streptomyces*.

56. A purified α subunit of an RNA polymerase of *Agrobacterium* or a portion thereof.

57. A purified α subunit of an RNA polymerase of *Agrobacterium* of claim 55 or a portion thereof wherein the amino acid sequence of said α subunit is depicted in Figure 2A (SEQ ID NO:1) or a portion thereof.

58. A purified α subunit of an RNA polymerase of *Agrobacterium* of claim 55 or portion thereof, wherein the α subunit of RNA polymerase is encoded by the nucleic acid is depicted in Figure 2B (SEQ ID NO:2) or a portion thereof.

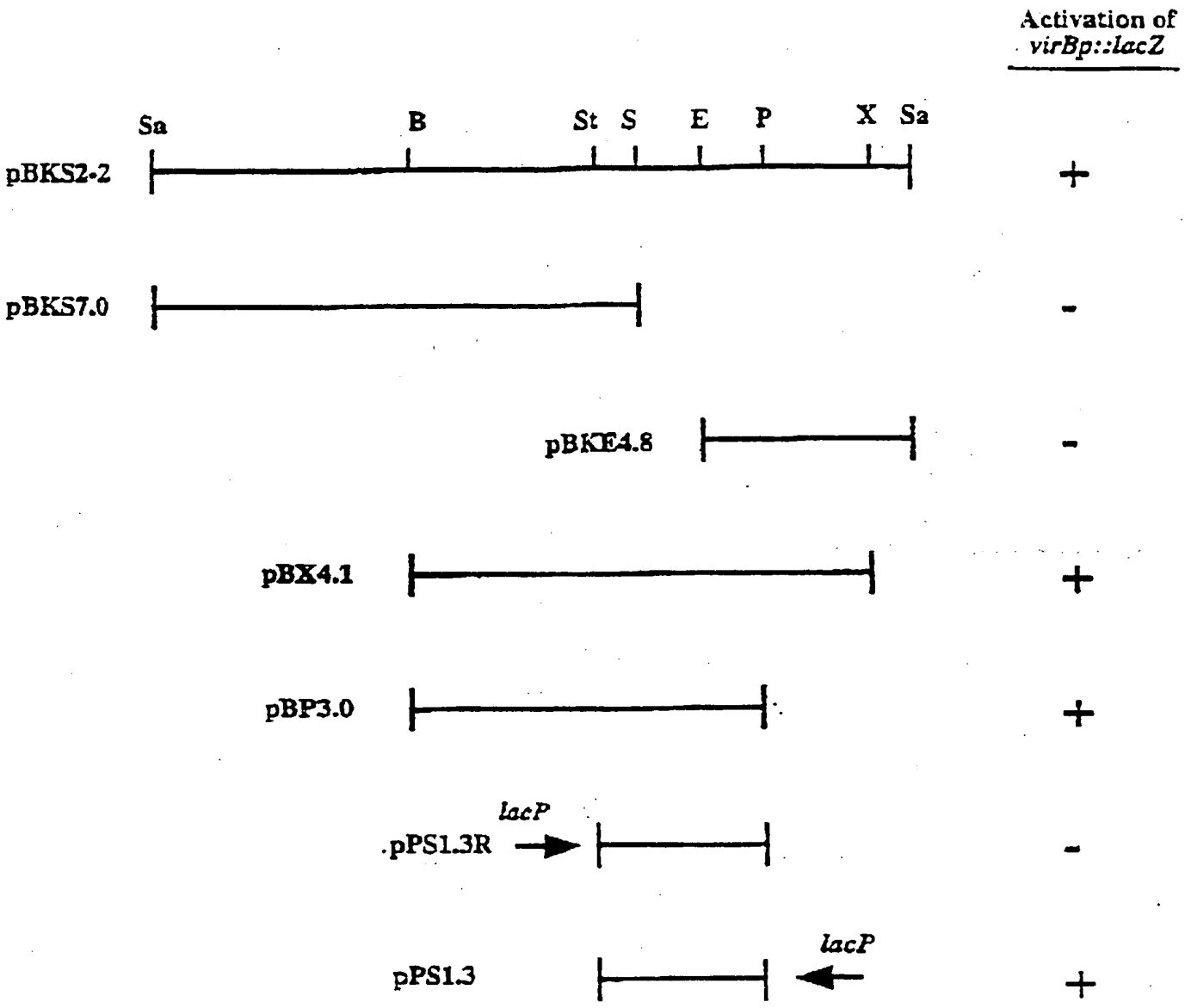

FIG. 1

FIG. 2A

MIQKNWQELIKPNKVEFTSSSRTKATLVAEPLERGFGLTLGNALRRVLLS 50

SLRGAAVTAVQIDGVLHEFSSIPGVREDVTDIVLNIKEIAIKMDGDDSKR 100

MVVRKQGPGSVTAGDIQTVGDIEILNPDHVICTL.DEGAEIRMEFTVNNG 149

KGYVPAE--RNRAEDAPIGLIPVDSLYSPVKKVSYKVENTREGQVLDYD 196

KLIMTIETNGSVSGEDAVAFAARILQDQLGVFVNFDEPQKEAEEESVTEL 246

AFNPALLKKVDELELSVRSANCLKNDNIVYIGDLIQKTEAEMLRTPNFGR 296

KSLNEIKEVLASMGLHLGMEVPAWPPENIEDLAKRYEDQY 336

FIG. 2B

1 aacacggcat gaagtcgctt gaagtcgaag tttgcggtcc gggttccggt cgtgaatcgg
61 cacttcgcgc tctgcaggct gccggtttca tgatcacttc cattcgcgac gccgatcccg
121 cacaacggtt gccgtccgcg caagaagcgc cgcgtctgac gcgaccgtgg ttcggaaatt
181 ccgcctttcc ttcggtctgg cggaattttc gtgtatctgg cgtgtgcgcg tcgatttcga
241 tcgacggact tgcgctcaag aacccactga tgaaccactg aattaggttc ctctcggtgt
301 tttcatgctc ggtccgtcac gattggatgg tggcggcgaa cggaaggttt aaagatgatt
361 cagaagaact ggcaggaact tatcaagccg aacaaggtcg agttcacctc gtccagccgc
421 accaaggcaa ctctggttgc cgagccgctg gagcgtggtt tcggtcttac cctcggcaac
481 gcgctgcgcc gcgttctgtt gtcttctctg cgtggtgccg ctgtaacggc cgtgcagatc
541 gacggtgtcc tgcacgaatt ctcctccatc cccggcgttc gggaagatgt gacggatatc
601 gtgctcaaca tcaaggaaat cgccatcaag atggatggtg acgattccaa gcgcatggtc
661 gtgcgcaagc agggtccggg ttcggtaacc gctggtgaca tccagacggt tggcgacatc
721 gagatcctga accccgacca cgtgatctgc acgctcgatg aaggcgctga aatccgcatg
781 gaattcaccg tcaacaacgg caagggttac gtaccggctg agcgcaaccg cgcggaagat
841 gcccctatcg gcctcattcc ggtggacagc ctctattctc cggtcaagaa agtgtcctac
901 aaggtggaaa acacccgtga aggtcaggtt ctcgactatg acaagctgat catgacgatc
961 gagaccaacg gttcggtttc cggcgaagac gccgttgcct tcgccgctcg cattcttcag
1021 gaccagctgg gcgtcttcgt caacttcgac gagccgcaga aggaagcaga agaagaatcg
1081 gttactgaac tcgcgttcaa cccggcgctt ctcaagaagg tcgacgagct cgaactgtca
1141 gttcgttcgg caaactgcct gaagaacgac aacatcgttt atatcggcga cctgatccag
1201 aagaccgaag ccgaaatgct ccgcacgccg aactttggtc gcaagtcgct gaacgaaatc
1261 aaggaagttc tcgcttccat gggtctgcac ctcggcatgg aagtgccggc atggccgcct
1321 gagaacatcg aagatctcgc aaagcgttac gaagaccaat actaacaaac aagaaggcag
1381 accttaaaga ctgcctttcc ccgtcaaaca gcagataagt catctgcatg tgccaggaaa
1441 cggcaggcct taaagaaggc acctgcgtag aaggagaata gcaatgcgcc acggtaattc
1501 aggccgcaag ctcaatagaa ccgccagcca ccgcaaggca atgtttgcca acatggctgc
1561 ttcgctcatc acccatgagc agatcgtcac caccttccg aaggcgaagg aaatccgtcc
1621 gatcgtcgag cgtctcgtga cgctgggcaa gcgcggcgac ctgcacgctc gtcgtcaggc
1681 gatctcgcag at

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A. tum.   MIQKNWQELIKPNKVEFTSSSRTKATLVAEPLERGFGLTLGNALRRVLLS 50
E. coli   -MQGSVIEELKPRLVDIEQVSSTHAKVTLEPLERGFGETLGNALRRILLS 49
          .*  .  * .**  *.    * * * .  ******** ********.***

A. tum.   SLRGAAVTAVQIDGVLHEFSSIPGVREDVTDIVLNIKEIAIKMDGDDSKR 100
E. coli   SMPGCAVTEVEIDGVLHEYSTKEGVQEDILEILLNLKGLAVRVQGKDEVI 99
          *. * *** *.*******.*.  **.**. .*.**.* .*... * *

A. tum.   MVVRKQGPGSVTAGDIQTVGDIEILNPDHVICTL.DEGAEIRMEFTVNNG 149
E. coli   LTLNKSGIGPVTAADITHDGDVEIVKPQHVICHLTDENASISMRIKVQRG 149
          . . * * * *** **   **.**. * **** * ** * * *   * *

A. tum.   KGYVPAE---RNRAEDAPIGLIPVDSLYSPVKKVSYKVENTREGQVLDYD 196
E. coli   RGYVPASTRIESEEDERPIGRLLVDACYSPVERIAYNVEAARVEQRTDLD 199
          .*****     .  .. *** . **. ****....* **  *  *  * *

A. tum.   KLIMTIETNGSVSGEDAVAFAARILQDQLGVFVNFDEPQKEAEEESVTEL 246
E. coli   KLVIEMETTGTIDPEEAIRRAATILAEQLEAFVDLRDVRQPEVKEEKPE- 248
          **.. .** *..  *.*.  ** ** .**  **.  . ..   .*   *

A. tum.   AFNPALLKKVDELELSVRSANCLKNDNIVYIGDLIQKTEAEMLRTPNFGR 296
E. coli   -FDPILLRPVDDLELTVRSANCLKAEAIHYIGDLVQRTEVELLKTPNLGK 297
           *.* **. **.***.******** . * *****.*.** *.*.*** *.

A. tum.   KSLNEIKEVLASMGLHLGMEVPAWPPENIEDLAKRYEDQY 336
E. coli   KSLTEIKDVLASRGLSLGMRLENWPPASIADE 329
          *** ***.**** ** ***  .  *** .* *
```

FIG. 3

FIG. 4A

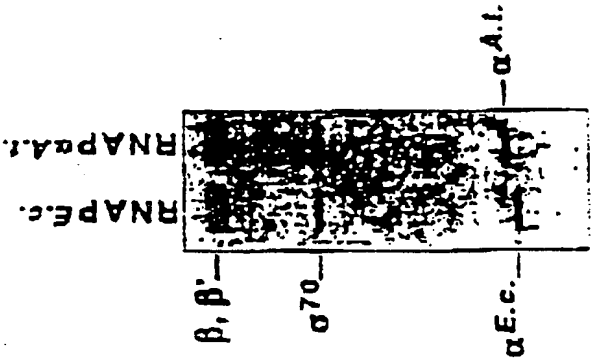
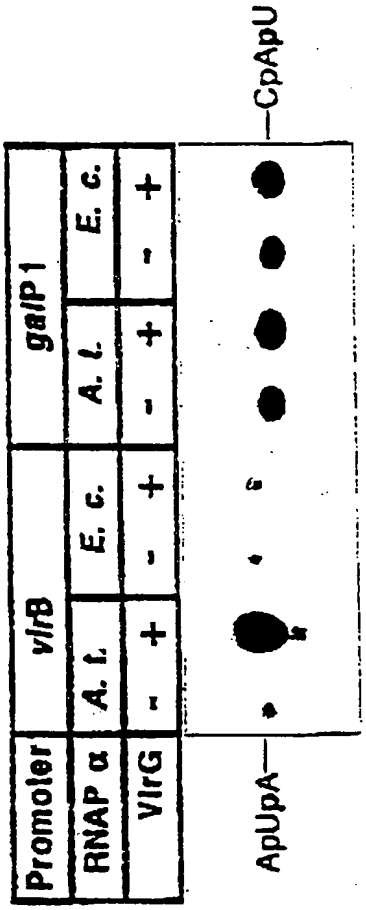
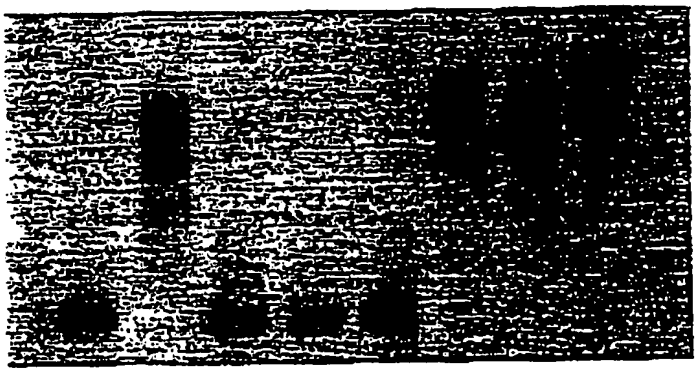


FIG. 4B



1 2 3 4 5 6 7 8

FIG. 5A



- - + + + + + + RpoA

- + - - - + + + VirG

FIG. 5B

1 2 3 4 5 6 7 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/10014

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :C12P 21/02; C12N 15/62; C07H 21/04; C12N 9/00
US CL :435/69.1, 183; 536/23.1, 23.4
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 183; 536/23.1, 23.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	LOHRKE et al. Transcriptional Activation of *Agrobacterium tumefaciens* Virulence Gene Promoters in *Escherichia coli* Requires the *A. tumefaciens* rpoA Gene, Encoding Alpha Subunit of RNA Polymerase. Journal of Bacteriology. August 1999, Vol. 181, No. 15, pages 4533-4539.	1, 2, 11-14, 21-24, 31-33, 35-56
A	STEFFEN et al. Hybrid *Bordetella pertussis-Escherichia coli* RNA Polymerases: Selectivity of Promoter Activation. Journal of Bacteriology. March 1998, Vol. 180, No. 6, pages 1567-1569.	11-14, 21-24, 31-33, 35-56

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 14 JUNE 2000	Date of mailing of the international search report 20 JUL 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer GERALD G. LEFFERS JR. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/10014

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BOYLAN et al. Gene Encoding the Alpha Core Subunit of *Bacillus subtilis* RNA Polymerase is Cotranscribed with the Genes for Initiation Factor 1 and Ribosomal Proteins B, S13, S11, and L17. Journal of Bacteriology. May 1989, Vol. 171, No. 5, pages 2553-2562, especially Figure 2.	11-14, 21-24, 31-33, 35-56
A	WO 98/24891 A1 (SCRIPTGEN PHARMACEUTICALS, INC.) 11 June 1998 (11.06.98), see entire document, especially pages 2-4.	11-14, 21-24, 31-33, 35-56

Form PCT/ISA/210 (continuation of second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/10014

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 3-10, 15-20, 57-58
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

No sequence listing or computer readable form of the sequence listing was submitted with this case. Also, the claims are drawn to sequences given in figures rather than to sequences given in a sequence listing and having sequence identifiers.

3. ☒ Claims Nos.: 25-30, 34
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/10014

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

EAST: DERWENT, JPO, EPO, USPAT; STN: MEDLINE, EMBASE, BIOSIS, CAPLUS
search items: inventor names, rpoA, map, rna polymerase, alpha subunit, hybrid, fusion, agrobacterium, tumefaciens, coli, escherichia, pseudomnas, bacilus, lactobacillus, rhizobium, mycobacterium, chlamydia, streptomyces

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

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International Bureau

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26 October 2000 (26.10.2000)

PCT

(10) International Publication Number
WO 00/63413 A1

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(21) International Application Number: PCT/US00/10014

(22) International Filing Date: 14 April 2000 (14.04.2000)

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60/129,682	16 April 1999 (16.04.1999)	US
60/134,206	14 May 1999 (14.05.1999)	US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier applications:

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US	60/134,206 (CIP)
Filed on	14 May 1999 (14.05.1999)

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(54) Title: AGROBACTERIUM TUMEFACIENS RPOA GENE

(57) Abstract: The present invention is directed to a method for expression of at least one heterologous gene in a host cell comprising transforming a host cell with at least one nucleic acid construct comprising a complete α subunit of an RNA polymerase or a portion thereof of a hybrid nucleic acid containing a portion of the α subunit of an RNA polymerase obtained from the same genus as the heterologous gene, and transforming the host cell, with at least one heterologous gene; and culturing the transformed host cell. The present invention further is directed to nucleic acid molecules used in the present method, vectors containing these nucleic acid molecules, and host cells containing the nucleic acid molecules. The nucleic acid encoding the α subunit of an *Agrobacterium* RNA polymerase and the corresponding amino acid sequence and portions thereof is disclosed.

AGROBACTERIUM TUMEFACIENS RPOA GENE

This application is a continuation-in-part of provisional application Serial No. 60/129,682 filed on April 16, 1999 and a continuation-in-part of provisional application Serial No. 60/134,206 filed on May 14, 1999, both of which are incorporated herein in their entirety by reference.

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The present invention relates to nucleic acid molecules and constructs containing these nucleic acid molecules and methods of using these constructs to express heterologous genes in hosts. In particular, the nucleic acid molecules of the present invention comprise a DNA sequence encoding at least a portion of the α subunit of the RNA polymerase (referred to herein as RNAP) obtained from the same genus source from which the heterologous genes were originally isolated. The nucleic acid constructs also can optionally comprise, if required for expression of the heterologous genes, at least one gene encoding a transcriptional regulator, also obtained from the same genus as the source of the heterologous genes.

BACKGROUND OF THE INVENTION

Although many host cell systems are well characterized and utilized to express a number of heterologous genes, there are still many genes that cannot be expressed in such host cell systems, as for example, *Escherichia coli* (*E. coli*). It is believed that the lack of expression in these host cell systems is a result of the promoter sequences of the heterologous genes not being recognized by the host RNAP. The present invention has solved this problem by showing that this block in expression can be overcome by co-expression of at least a portion of the *rpoA* gene product, the gene that encodes the α subunit of the RNAP, obtained from the same genus as the source of the heterologous gene that is desired to be expressed in the new host cell. The co-expressed α subunit of the RNAP from the same genus as the source of the heterologous gene combines with the other

subunits of the RNAP, β, β', and σ, to form a functional RNAP. Additionally, if transcriptional regulators, such as transcriptional activators or transcriptional repressors, or additional other subunits of the RNAP, such as β, β' or σ, are required to obtain expression of the heterologous gene, then these additional components also are obtained from the same genus as the source of the heterologous gene. Since two different *rpoA* genes are present in a single host cell, there will be various combinations of RNAPs present in the host cell (1) RNAP containing two α subunits of the same genus as the host (2) RNAP containing two α subunits from the same genus source as the heterologous gene, and (3) RNAP containing one α subunit from the same genus as the host and one α subunit from the same genus source as the heterologous gene. A heterologous gene is intended to mean a gene that is not from the same source as the host cell.

Agrobacterium tumefaciens is a Gram-negative soil bacterium which is the causative agent of Crown Gall disease, affecting primarily dicotyledonous plant species (reviewed in 18, 62). The pathogen incites production of the characteristic tumor through the transfer of a piece of DNA (T-DNA) from the Ti (Tumor inducing) plasmid into susceptible plant cells, with subsequent integration into the host genome. The T-DNA contains genes that direct the biosynthesis of auxin and cytokinin in infected cells (1, 57), resulting in uncontrolled cell division leading to production of the characteristic tumor. The T-DNA also contains genes for the biosynthesis of unique compounds called opines which the bacterium can utilize as a carbon and nitrogen source (39).

Successful transfer of the T-DNA is dependent on the coordinated expression of virulence *(vir)* genes located on the Ti plasmid but separate from the T-DNA. Expression of *vir* genes occurs in response to certain phenolic compounds released from wounded plants (54). This expression is augmented by monosaccharides *(5,* 52), and an acidic pH (38) which are characteristics of plant wound sites. Expression of *vir* genes requires *virA* and *virG,* which are members of the family of two component regulatory systems (60). VirA is an inner membrane associated histidine protein kinase which autophosphorylates in response to the environmental signals (19, 28). The phosphate moiety is subsequently transferred to the aspartate residue of VirG, which in turn activates transcription from promoters containing a specific 12 base pair sequence called the *vir* box, present in the promoters of all *vir* genes (29, 44). In addition to *virA* and *virG*, other chromosomally

encoded genes have been identified in *A. tumefaciens* that have been shown to modulate virulence gene expression either directly or indirectly (12, 15, 20, 61).

The use of *E. coil* as a heterologous host in which to study the regulation of *A. tumefaciens* virulence genes and the mechanism of T-DNA transfer constitutes an ideal model system given the degree of characterization at both the biochemical and genetic level. However, all previous attempts to reconstitute *vir* gene expression in *E. coil* have not been successful. Possible explanations for the lack of *vir* gene expression include the presence of unidentified regulatory genes in *A. tumefaciens* required for *vir* induction, and/or that *E. coli* may contain specific repressor(s) of *vir* gene induction.

A characteristic of *vir* gene promoters is the absence of a strong -35 sequence (10). Dnase I footprinting studies have shown that VirG protects a region extending into where the -35 consensus sequence should be (29, 44). It has been suggested that binding of VirG may functionally replace the -35 consensus sequence allowing transcription to occur. This situation is similar to Class II CAP-dependent promoters, in which the CAP binding site overlaps with the -35 sequence. Studies have demonstrated that transcription at Class II CAP-dependent promoters requires interaction between CAP and the α subunit (*rpoA*) of RNAP (42, 49, 64). Many transcriptional factors from *E. coli* are known to require interaction with the α subunit of RNAP , including FNR (59), GaiR (9), MarA (24), Mer R (7), MetR (23), OxyR (56), OmpR (21), Rob (26), SoxS (25), and TyrR (34). Analysis of the α subunit from *E. coli* indicates the presence of two independent domains, the N-terminal domain and the C-terminal domain (21, 27, 63). The N-terminal domain (NTD) is involved in the assembly of the core polymerase, while the C-terminal domain (CTD) is involved in interaction with certain transcriptional regulators (7, 9, 21, 23, 24, 25, 26, 32, 34, 47, 56, 59). Recently, interaction between the NTD and CAP at Class II CAP-dependent promoters has been demonstrated (42,49).

The present methods of expressing heterologous genes are particularly useful to express multiple genes or operons in a metabolic pathway by co-expressing the *rpoA* gene product obtained from the same natural hosts from which the genes or operons were originally isolated with the multiple genes. The present method provides an advantage over existing multiple gene expression systems by eliminating the need to separately isolate each gene in the metabolic pathyway and link each gene to a promoter that is functional in the host. Expression of heterologous genes for an entire metabolic pathway in hosts is

particularly useful to produce particular metabolites, such as pharmaceuticals, food supplements, chemical products or fine chemical products.

The present invention further provides nucleic acid molecules and methods whereby heterologous genes encoding metabolites in an entire metabolic pathway can be introduced into hosts so that these hosts possess specific catabolic and/or metabolic characteristics that allow the hosts to grow or express gene products under specific environmental conditions. These hosts are useful in bioremediation, bioassays and biocontrol studies where manipulation of natural or symbiotic bacterial flora is desired, such as in the control of parasites or insects where bacterial symbiants are involved.

The present invention broadly relates to the expression of heterologous genes in a host using nucleic acid molecules comprising a gene encoding the complete α subunit of the RNAP obtained from the same genus as the source of the heterologous genes or at least a portion of the α subunit of the RNAP. The host and the heterologous genes to be expressed may be prokaryotic or eukaryotic in origin, however, prokaryotic hosts are preferred. The present invention also relates to a hybrid nucleic acid molecule encoding a hybrid α subunit of RNAP, where a first nucleic acid comprises at least a portion of the *rpoA* gene encoding the α subunit of an RNAP obtained from the same genus as the source of the host cell and a second nucleic acid comprises at least a portion of the α subunit of an RNAP obtained from the same genus as the heterologous gene.

The present invention specifically discloses the *rpoA* gene from *Agrobacterium* encoding the α subunit of the RNAP and the corresponding amino acid sequence. Portions of both the *rpo*A gene and the α subunit of RNAP of *Agrobacterium* also are encompassed by the present invention, particularly the sequences disclosed in Figs. 2A and 2B. The *rpo*A gene is useful in methods of expressing *Agrobacterium* genes in hosts, particularly prokaryotic host cells. Prior to the present invention, it was not possible to express *Agrobacterium* genes, particularly *Agrobacterium* virulence genes, in *E. coli*. Therefore, the present invention provides a method for studying *Agrobacterium* genes and their regulation.

The invention also relates to genes encoding hybrid *rpoA* genes that comprise at least a portion of an *rpoA* gene from the same genus as a heterologous gene which is expressed, such as the *Agrobacterium rpoA* gene, with the remainder of the *rpoA* gene obtained from the same genus as the host cell in which the heterologous gene is expressed.

SUMMARY OF THE INVENTION

The present invention is directed to an isolated nucleic acid molecule encoding the complete α subunit of an RNAP of *Agrobacterium* or at least a portion thereof. Specifically, the present invention is directed to an isolated nucleic acid molecule encoding the complete α subunit of an RNAP of *Agrobacterium*, in which the amino acid sequence of the α subunit is depicted in Figure 2A or at least a portion of this sequence. More specifically, the present invention is directed to an isolated nucleic acid molecule comprising the complete nucleic acid sequence as depicted in Figure 2B or at least a portion thereof. These isolated nucleic acid molecules encoding at least a portion of the α subunit of an RNAP of *Agrobacterium* also are optionally linked to a promoter that is functional in the host in which these molecules are expressed. Further, the present invention is directed to vectors containing these nucleic acid sequences or molecules. The vectors are useful in transforming host cells for the expression of heterologous gene(s). The invention is also directed to host cells that are transformed with the nucleic acid molecules described herein. The nucleic acid molecules useful to transform the host cells are operably linked to a promoter and encode an α subunit of an RNAP or at least one heterologous protein.

The present invention is also directed to a hybrid nucleic acid molecule for the expression of at least one heterologous gene in a host cell comprising a first nucleic acid sequence encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the host cell and a second nucleic acid sequence encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the source of the heterologous gene; and vectors containing the hybrid nucleic acid molecule.

The present invention is further directed to a method of expressing at least one heterologous gene in a host cell comprising transforming a host cell with a vector comprising a nucleic acid molecule encoding a complete α subunit of RNAP from the same genus as the source of the heterologous gene or at least a portion thereof, and also transforming the host cell with a vector comprising at least one heterolgous gene. The nucleic acid molecule and the heterologous gene(s) also may be contained in one vector but preferably these sequences are contained within two or more vectors. In a further embodiment, the present invention is also directed to a method of expressing at least one heterologous gene in a host cell comprising transforming a host cell with a vector comprising a hybrid nucleic acid molecule comprising a first nucleic acid sequence

encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the host cell and a second nucleic acid sequence encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the source of the heterologous gene, and transforming the host cell with a vector comprising at least one heterologous gene; and culturing the transformed host cell under conditions where the heterologous gene is expressed in the host cell. The host cell also may be transformed with a single vector containing the nucleic acid molecule encoding the complete α subunit of the RNAP, a portion thereof or a hybrid molecule comprising a portion of the α subunit of the RNAP, and with the heterolgous gene(s) or α subunit of the RNAP peferably, two or more vectors may be used. The nucleic acid molecules encoding the α subunit and the heterologous gene(s) may be in separate vectors for transformation of the host cell. The host cell can optionally be transformed with at least one gene encoding a heterologous transcriptional regulator obtained from the same genus as the source of the heterologous gene. The heterologous gene may comprise multiple genes or operons in the same metabolic pathway.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Physical and genetic map of recombinant plasmids carrying the rpoA gene from *A. tumefaciens* is depicted. Abbreviations: B, BamHI; E. FcoRI; P, PstI; Sa, Sm3A; St, Sttd, X, XhoI Location and direction of *rpoA* and *lac* promoter for constructs pPS1.3R and pPS1.3 are indicated by arrows.

Figure 2A. The amino acid sequence of the *A. tumefaciens* α subunit of the RNAP is depicted.

Figure 2B. The *A. tumefaciens rpoA* gene encoding the *A. tumefaciens* α subunit of the RNAP is depicted. The actual coding sequence for the α subunit is located between nucleotide 355 (atg) and nucleotide 1356 (taa).

Figure 3. Amino acid sequence alignment of the *A. tumefaciens and* E. coli rpoA gene products. Identical amino acid residues at a given position are marked by an asterisk, and conserved substitutions are marked by a dot. Gaps introduced to allow optimal alignment are designated by a dash. The bolded nucleotides show three highly conserved regions between the two amino acid sequences.

Figure 4 A and B. Assembly of His-rpoA into RNAP, and *in vitro* transcription assays. Figure 4A - SDS-PAGE analysis of reconstituted RNAP containing *E. coli* β, β',

σ^{70} subunits and His-RpoA from *E. coli* or *A. tumefaciens* and stained with Coomassie brilliant blueB. Figure 4B - *In vitro* transcription from *virB* and *galP*1 promoters in the absence and presence of $VirG^{con}$. The figure shows the result of a multiround transcription reaction employing reconstituted RNAP containing His-rpoA from either *E. coli* or *A. turnefaciens*. Where indicated, 2 pmol of $VirG^{con}$ was added to the reaction.

Figure 5. Protein-protein and protein-DNA interactions between RpoA, $VirG^{con}$ and the *virB* promoter. The figures show electrophoretic mobility shift assays of [^{32}P]-labeled *virB* promoter containing purified $VirG^{con}$ and His-RpoA from *E. coli* in Figure 5A or *A. tumefaciens* in Figure 5B Lane 1: Promoter only, Lane 2: Promoter + $VirG^{con}$, Lane 3: Promoter + 150 nM RpoA, Lane 4: Promoter + 300 nM RpoA, Lane 5: Promoter + 600 nM RpoA, Lane 6: Promoter + $VirG^{con}$ + 150 nM RpoA, Lane 7: Promoter + $VirG^{con}$ + 300 nM RpoA, Lane 8: Promoter + $VirG^{con}$ + 600 nM RpoA.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Although many well characterized host cells, such as *E. coli*, provide advantages as an expression host cell, it has not been possible to express the genes of many bacteria in these host cells because the promoter sequences of the heterologous genes are not recognized by the host cell RNAP. The present invention provides a method of overcoming this block to expression by co-expressing the α subunit of the RNAP from the same genus as the source of the heterologous genes that are to be expressed in the host,and optionally co-expressing the transcriptional activator from the same genus as the source of the heterologous genes. The success of this method has been demonstrated by showing the expression of the *Agrobacterium tumefaciens* virulence (*vir*) gene operons in *E. coli*. In this specific example, expression of these genes was shown to require not only the *vir* gene specific transcriptional regulator but also the *rpoA* gene from *A. tumefaciens*. The *A. tumefaciens* α subunit of the RNAP is able to assemble with the other subunits (β, β' and σ subunits) of the *E. coli* RNAP to form a functional polymerase which specifically recognizes the *Agrobacterium* genes in the presence of the *Agrobacterium* transcriptional regulator, specifically an activator. The results show that this interaction is genus specific, in that, the transcriptional activator from *Agrobacterium* can only efficiently interact with the rpoA gene product from *Agrobacterium* and not with that of *E. coli*.

Conventional understanding of gene expression in prokaryotes emphasizes the importance of the transcriptional regulator, either an activator or a repressor. A transcriptional activator is a protein that is needed to turn on the expression of target genes either directly or indirectly whereas repressors are proteins that inhibit the transcription of target genes either directly or indirectly. In the present invention, it is shown that the α subunit of the RNAP is the most critical factor in activating bacterial genes, especially those requiring transcriptional regulators. For example, to express heterologous genes or operons from organism A in organism B, at least a portion of the *rpoA* gene from organism A is also needed to be co-expressed in organism B. Likewise if transcriptional regulators are required for expression of heterologous genes, the genes encoding the transcriptional regulator(s) from organism A should also be used to transform the host because these regulators can most efficiently interact with RpoA from the same organism A but not as well or at all with that of organism B.

The present method is particularly advantageous when it is used to express particular heterologous metabolic pathway genes in hosts for bioremediation/biocontrol purposes or production of particular metabolites. This approach becomes much more practical than fusing each gene or operon under the control of a known promoter that is functional in the host.

Expression of genes for an entire metabolic pathway in heterologous hosts is useful to produce particular metabolites or to confer to the host specific catabolic or metabolic characteristics that now enables the host to grow or express gene products under specific environmental conditions.

An example of the utility of the present method is shown by the production of a plant growth promoting molecule, indole-3-acetic acid, from *Azospirillum brasilense*, that requires at least 3 biosynthetic genes. *A. brasilense* also possesses multiple genes that encode products that fix nitrogen (65). The multiple genes responsible for encoding these products and the *rpoA* gene are introduced via one or more vehicles, such as plasmids or cosmids, into a host that is applied to a plant and that expresses the products of the multiple genes. The expressed plant growth promoting molecules and nitrogen fixing molecules affect the growth of the treated plant.

Further *Rhizobium*, *Bradyrhizobium*, *Azorhiobium* and *Sinorhizobium* are other examples of nitrogen-fixing bacteria, each involving at least two dozen genes for nodulation

(symbiosis) and multiple genes for nitrogen fixation. These bacteria, as well as other bacteria that produce useful products for plants, may also be the source of the heterologous genes and the *rpoA* gene.

The present invention can also be utilized to express gene product(s) in a catabolic pathway that degrades or breaks down toxic or environmentally hazardous products. For example, a *Pseudomonas* species strain ADP expresses at least four genes that are involved in the degradation of a herbicide, Atrazine (66). These genes are introduced and expressed in a host by applying the host to areas where this herbicide is present, resulting in the degradation of the herbicide on site.

Further, the present invention is useful in providing a biocontrol system that includes a host that expresses heterologous gene products, that for example, kill disease producing organisms on a plant, animal, human or a surface that will support growth of the host. *Pseudomonas chloroaphis* strain 30-84 expresses at least three antifungal metabolites, phenazine-1-carboxylate, 2-hydroxyphenazine-1-carboxlylic acid and 2-hydroxyphenazine that involves four biosynthetic genes and 2 regulatory genes (67). These genes may be introduced into a plant bacteria, such as *Rhizobium*, that can be applied to a plant to control the growth of fungi on the plant.

The present invention also is useful as an *A. tumefaciens* gene delivery system in heterologous hosts for potential gene delivery or gene therapy systems of *A. tumefaciens* genes to various hosts.

The present invention also can be used to express heterologous genes in *A. tumefaciens* to alter its characteristics (e.g. host specificity) for a broader application of the *A. tumefaciens* mediated gene delivery system.

The present invention also is useful to prepare host cells containing an *rpoA* gene that is heterologous to the host cell, such as Escherichia. This host cell is useful for studying changes in gene expression patterns in this cell.

The present invention can additionally be used to express particular sets of heterologous genes, including multigene operons/stimulons or genes encoding multi-subunit protein complexes, such as proteosomes or gene libraries in hosts as targets for drug screening and/or diagnostics.

The present invention discloses an isolated nucleic acid molecule encoding the complete α subunit of an RNAP of *Agrobacterium* or at least a portion thereof. More

specifically, an isolated nucleic acid molecule encoding the complete α subunit of an RNAP of *Agrobacterium*, where the amino acid sequence of the α subunit is depicted in Figure 2A is disclosed or at least a portion thereof. The portion can be a length of amino acid sequences that is sufficient to express any heterologous gene in the host cell into which it is introduced. The portion of the α subunit of the RNAP also should be of a sufficient length to provide at least one of the following functions: 1) bind to DNA in the promoter region 2) interacts with the other subunits of RNAP; i.e., β, β' and σ to form a functional RNAP, and 3) interacts with the transcriptional activator. More specifically, an isolated nucleic acid molecule comprising at least a portion of the nucleic acid sequence as depicted in Figure 2B and that encodes at least a portion of the amino acid sequence of Figure 2A is disclosed. The nucleic acid molecule (rpoA) encoding the *Agrobacterium* α subunit of the RNAP or a portion of the nucleic acid molecule is useful in the expression of *Agrobacterium* genes in any prokaryotic host cells.

The present invention is also directed to a nucleic acid molecule encoding a hybrid α subunit of RNAP where the isolated nucleic acid molecule is inserted in a vector and is useful in expressing at least one heterologous gene in a host cell comprising a first nucleic acid sequence encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the host cell and a second nucleic acid sequence encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the source of the heterologous gene. This isolated nucleic acid molecule is operably linked to a promoter so that it is under the control of a promoter that is functional in the host cell. For example, the *E. coli lac* or *trp* promoters are useful to express the *rpo*A gene in an *E. coli* host cell. Promoters that function in numerous host cells are well known to persons skilled in the art and can be selected based upon the host cell selected. The second nucleic acid sequence is obtained from any eukaryotic or prokaryotic host cell, and more particularly from a prokaryotic cell, including Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria. More specifically, the bacteria are selected from the genus *Agrobacterium*, *Mycobacterium*, *Chlamydia*, *Pseudomonas* or *Streptomyces*. More preferably, it is obtained from *Agrobacterium*, and most preferably the second nucleic acid sequence encodes the complete amino acid sequence as depicted in Figure 2A or a portion thereof, with the sufficient length of this portion discussed above. The portion of the of the *rpoA* gene can be from

any position in the gene; i.e., that is from the 5' or the 3' end of the gene, which encodes the N-terminus and C-terminus of the α-subunit, respectively. Most preferably, the nucleic acid molecule portion encodes at least amino acid residues 157 to 336 as depicted in Figure 2A. The nucleic acid molecule can encode as few as 8 consecutive amino acid residues of any portion of Figure 2A. Specifically, a portion comprising at least the amino acid residues 157 to 336, or of less than amino acid residues of 157 to 336, and more specifically amino acid residues 329 to 336 are useful in the present invention. The first nucleic acid sequence is obtained from any bacteria but more preferably from the genus *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*.

The present invention is directed to a method of expressing at least one heterologous gene in a host cell comprising transforming a host cell with a nucleic acid construct comprising a first nucleic acid sequence encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the source of the host cell, and a second nucleic acid sequence encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the source of the heterologous gene; and with at least one heterologous gene; and culturing the transformed host cell under conditions wherein the heterologous gene is expressed in the host cell. The method further comprising transforming the host cell with at least one gene encoding a heterologous transcriptional regulator obtained from the same genus as the source of the heterologous gene. The heterologous gene may comprise multiple genes or operons in the same metabolic pathway that catalyze the production of a product.

The host cells useful in the present method are prokaryotic cells or eukaryotic cells. Preferably, the host cell is selected from a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, aerobic bacteria, acid-fast bacteria, mycoplasma, anaerobic bacteria, and facultative bacteria. Preferably, the host cell is obtained from the genus *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*. Eukaryotic cells may be selected from known mammalian cells that can be grown in cell culture, such as CHO, BHK, COS.

The present invention also is directed to a method of expressing at least one heterologous gene in a host cell comprising transforming the host cell with a vector comprising a nucleic acid molecule encoding a complete α subunit of an RNA polymerase obtained from the same genus as the heterologous gene or a portion of the α subunit of the

RNA polymerase, and also transforming the host cell with at least one vector comprising at least one heterologous gene; and then culturing the transformed host cell under conditions where at least one of the heterologous genes is expressed in the host cell. The method further comprises transforming the host cell with at least one gene encoding a heterologous transcriptional regulator obtained from the same genus as the source of the heterologous gene. The transcirptional regulator is a transcriptional activator that interacts with the α subunit of the RNAP to enhance the expression of the heterologous gene. The heterologous gene may comprise multiple genes or operons in the same metabolic pathway.

Preferably, the host cell is transformed with two vectors, one containing the *rpoA* gene or a portion thereof and one containing at least one heterologous gene. However, the host cell may be transformed with a single vector containing the *rpoA* and heterologous genes, but more than two vectors can be used to introduce the genes into the host cell depending on the number of genes and the size of the nucleic acid to be introduced.

The host cell may be a prokaryotic cell or a eukaryotic cell. Preferably the host cell is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria. More preferably the host cell is from the genus *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*. Most preferably the host cell is an *Escherichia*.

The method is useful for the expression of at least one heterologous gene, where the genus of the source of the heterolgous gene is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, aerobic bacteria, anaerobic bacteria, and facultative bacteria. More preferably the genus of the source of the heterologous gene is *Agrobacterium*, *Mycobacterium*, *Chlamydia*, *Pseudomonas* or *Streptomyces*, and most preferably, the genus is obtained from *Agrobacterium*.

The method utilizes a nucleic acid molecule that encodes the α subunit of the RNAP the amino acid sequence as depicted in Figure 2A or at least a portion of the amino acid sequence depicted in Figure 2A. The portion of the amino acid sequence useful in the present method may be from the N-terminus or C-terminus of the α subunit of the RNAP but preferably comprises at least amino acid residues 157 to 336 of the amino acid sequence depicted in Figure 2A or the portion of the amino acid sequence may comprise less than amino acid residues 157 to 336 of the amino acid sequence depicted in Figure 2A. The

portion of the amino acid sequence should comprises at least 8 consecutive amino acid residues of Figure 2A. More specifically, the portion of the amino acid sequence as depicted in Figure 2A comprises amino acid residues 329 to 336.

The present invention also encompases a purified α subunit of an RNAP of *Agrobacterium*. More specifically, the purified α subunit of an RNAP of *Agrobacterium* of the present invention is the amino acid sequence depicted in Figure 2A. The invention is also intended to encompass portions of the purified α subunit of an RNAP disclosed in Figure 2A. More specifically, the purified α subunit of an RNA polymerase of *Agrobacterium* or a portion thereof is encoded by the nucleic acid is depicted in Figure 2B or a portion thereof.

A *rpoA* gene from the same genus as the source of a heterologous gene encoding the α subunit from this source is obtained by methods well known to persons skilled in the art. For example, this gene can be obtained: (1) by complementation of the a temperature sensitive *rpoA* mutant strain of a known organism, i.e. E.coli as discussed below in the preferred embodiment; (2) by Southern hybridization based on DNA sequence homology; (3) by Western blot based on cross reactivity of an antibody against a known RNAP α subunit; and (4) by PCR amplification based on conserved DNA sequences among known rpoA genes. For their expression, the *rpoA* genes are fused behind or operably linked to a promoter that is known to be operable in the host. The fusion is either a transcriptional fusion or translational fusion. Many standard methods are well known to persons skilled in the art and can be utilized to carryout these methods (See reference 68 which is herein incorporated in its entirety by reference.)

The following specific examples set forth below serve to further illustrate the present invention in its preferred embodiments, and are not intended to limit the present invention to these examples.

EXAMPLES

Bacterial strains, plasmids and media.

All strains and plasmids used or constructed are listed in Table 1. Bacterial strains were grown in either LB medium (40), Mannitol Glutamate Luria salts (MG/L) medium (58), or Induction medium containing 1% glucose (61) at 28°C. Induction medium was used for attempts to reconstitute wild type *vir* gene induction, while MG/L and LB medium

were utilized for strains containing *virG*^{con} (i.e. pSY215 and pLG2). When appropriate, media was supplemented with ampicillin (100 μg/ml), gentamycin (20 μg/ml), kanamycin (50 μg/ml) and tetracycline (20 μg/ml) for *E. coli* and carbenicillin (100 μg/ml), gentamycin (100 μg/ml), kanamycin (100 μg/ml) and tetracycline (5 μg/ml) for *A. tumefaciens*. For determinations of β-galactosidase activity, 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside (X-Gal) and isopropylthio-β-galactoside (IPIG) were included at a final concentration of 75 μg/ml and 1 mM, respectively. Acetosyringone (Sigma) and glucose was included, when necessary at 200 μM and 1%, respectively. For induction assays, *E. coli* stain MC4100 containing constructs indicated in Table 1 were grown to stationary phase in 5 ml of appropriate medium, containing antibiotics as required. These overnight cultures were used to inoculate 125 ml flasks containing 30 ml of identical medium using 0.5 ml as an inoculum. The cultures were incubated at 28°C with shaking for 16 hours and assayed for β-galactosidase activity according to the method of Miller (40). For reconstitution of virulence gene expression in *E. coil,* plasmid constructs pSY204, pLG2, pGP159 and pSL107 were introduced into MC4100 by electroporation. Construct pH098 containing *lac-driven A. turnefaciens rpoA* was then introduced into these strains and initially screened on LB medium (pSY204 and pLG2) or induction medium (pGP159 and pSL107) containing appropriate antibiotics, 1 mM IPTG, 75 mg/mi X-Gal and 200 μM acetosyringone. Induction assays for these strains were carried out as described above.

Isolation and subcloning of *rpoA* locus

A cosmid clone library of *A. tumefaciens* strain A136, a derivative of strain C58 lacking the Ti plasmid, in pVK102 was described previously (8). The cosmid clones were transformed into *E. coli* strain MC4100 containing plasmid pSY215. Transformants were initially screened for the development of a blue color on induction media containing X-Gal, indicating expression of the *virBp::lacZ* fusion. A cosmid clone, designated pBK2, was isolated by this procedure and was used for subsequent subcloning attempts, as outlined in Figure 1.

DNA sequencing was performed by PCR mediated Taq DyeDeoxy Terminator Cycle sequence on an Applied Biosystems model 377 DNA sequencer. The GCG sequence analysis software package (Genetics Computer Group, Madison, WI) and the BLAST software package (2) were used for all DNA and protein sequence analysis. The complete

nucleotide sequence of the 1.3 Kb PstI-StuI DNA fragment of pPS1.3 was determined on both strands. The *rpoA* sequence has been deposited into GenBank (accession # AF111855)

Overexpression and purification of proteins

For overproduction of the RpoA proteins, *rpoA* genes from *E. coli* and *A. tumefaciens* were PCR amplified from the MC4100 chromosome and plasmid pBX4.1 (Figure 1), respectively, using primers designed to contain a *BamHI* restriction site. [*E. colt* l)5'-CCA AAG AGA GGA TCC AAT GCA GGG-3', 2) 5'-CCT TAA CCT GGG ATC CGG TTA CTC G-3'; *A. tumefaciens* 1) 5'-GGA AGG ATC CAA GAT GAT TCA GAA GA-3', 2) 5'-CCT GGAA TCC TGC AGA TGA CTT ATC TG-3']. The PCR products were initially cloned into PCR2.1TOPO (Invitrogen, Inc.), and then subcloned into pQE vectors (Qiagen) to generate pECH4 and pZL-2, containing N-terminal His-tagged fusions to RpoA of *E. colt* and *A. tumefaciens,* respectively.

The His-tagged RpoA proteins of *A. tumefaciens* and *E. coli* were prepared from *E. coli* strain SG13009 (pREP4) containing pZL-2 or pECH4, respectively. Cells were grown at 37°C in 500 ml of L-Broth containing selective antibiotics to an OD_{60Q} of 0.3, and then induced with 1 mM IPTG for 3 hours at 37°C. Cells were harvested, resuspended in 20 ml of binding buffer (20 mM Tris-HCl pH7.9, 500 mM NaCl, *5%* Glycerol) and lysed by sonication. The lysates were cleared by low speed centrifugation and loaded on a Fast Flow Chelating Sepharose Column charged with Ni^{2+} (Pharmacia, Inc.). The column was attached to a Waters 650 Advanced Protein Purification System, washed extensively with loading buffer containing 25 mM imidazole, and bound protein eluted with buffer containing 100 mM imidazole. Fractions containing greater than 90% RpoA were precipitated by addition of ammonium sulfate to 75% saturation. The resulting pellet was dissolved in a storage buffer (20 mM Tris pH7.9, 40 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 50% Glycerol) and stored at 20°C.

Recombinant β β' and σ^{70} subunits of *E. coli* RNAP were each purified from overexpression strains as described previously (16, 51). Induction was initiated with 1 mM IPTG at 37°C for *3* hours and the proteins present in inclusion bodies were then purified according to Tang (55). The constitutively active VirG protein ($VirG^{con}$) was purified from *E.* coli as described previously (30).

RNAP holoenzymes were reconstituted from individually purified *E. coli* β β' and σ^{70}, and either *E. coli* or *A. tumefaciens* RpoA as described (3). The molar ration of α, β

and β' in the reconstitution reactions was 1:4:8. After reconstitution and thermoactivation in the presence of σ^{70}, RNAP preparations were further purified by gel exclusion chromatography on a Superose-6 column (Pharmacia) and ion exchange chromatography on a Resource Q column (Pharmacia). The purified RNAP holoenzymes were concentrated by filtration through a C-100 concentrator (Amicon, Inc.) to ~1 mg/ml, and stored in 50% glycerol storage buffer at -20°C.

In *Vitro* Transcription Assays

For analysis of abortive initiation, 2 pmol of recombinant RNAP holoenzyme containing either *E. coli* or *A. tumefaciens* RpoA was incubated with 10 pmol of template DNA in 10 μl of transcription buffer (40 mM Tris-HCl pH7.9, 40 mM KCl, 10 mM $MgCl_2$) for 10 minutes at 23°C in the presence or absence of the $VirG^{con}$ protein (2 pmol). As templates, a 130 bp EconRI-HindIII fragment of plasmid pAA121 containing the ga/Pl promoter (-63 to +44) or a 380 bp PCR product of plasmid pSM243cd (Stachel 1985) containing the virB promoter (-248 to +81) were used (4, 10). Abortive initiation reactions were initiated by addition of 0.5 mM initiating dinucleotide (CpA for ga/Pl promoter and ApU for virB promoter) and 0.5 μM [α-^{33}p] UTP (ga/Pl) or [α-^{33}p] ATP (virB) (3000Ci/mmol). After 25 incubation at 37°C, reactions were terminated by the addition of an equal volume of urea loading buffer. The reaction products were resolved on urea-PAGE (20% polyacrylamide, 19:1 acrylamide:bis) and visualized by autoradiography.

Mobility shift assays

Electrophoretic mobility shift DNA binding assays were carried out using a PCR amplified *virB* promoter labeled with (α-^{32}P] dCTP. PCR reactions included 20 μCi (α-^{32}P]dCTP, plasmid pSM243cd as template, Primer 1:5'-TTC CAC GGT GAC GCA TCG AAT G-3', and Primer 2:5'-CCC CGA TCT CTT AAA CAT ACC TTA TCT CC-3'. Unincorporated nucleotides were removed using the Wizard™ PCR Preps DNA Purification System kit from Promega. Mobility shift reaction mixtures contained 1600 cpm ^{32}P-labeled *virB* promoter, 50 mM KCl, 20 mM Tris-HCl pH 7.0, 10 mM $MgCl_2$, 1 mM DTT, 10% Glycerol and 20 μg/ml Herring sperm DNA. Where indicated, 1.5 μM VirG was added, which was sufficient to saturate all VirG binding sites on the promoter, and His-RpoA was added at 150-500 nM final concentration. Reactions were incubated for 30 minutes at 22°C, loaded onto 6% polyacrtlamide, 10% glycerol vertical slab gels in

0.5X TBE buffer (46) and electrophoresed at 20V/cm at 4°C for 2 hours. Following electrophoresis the gels were dried and autoradiographed overnight at room temperature.

Identification of an *A. tumefaciens* gene that is required for *vir* gene expression in *E. coli*

To reconstitute *vir* gene induction in *E. coli,* plasmid construct pGP159 containing *virA* and *virG* under the control of their native promoters and a *lacZ* reporter gene fused downstream of the *virB* promoter *(virBp::lacZ* fusion) was introduced into *E. coli* strain MC4100. The resulting strain failed to activate transcription of the fusion in response to acetosyringone *(AS).* The inability of pGP159 to activate transcription may have been due to a lack of expression of the *virA/virG* genes, which are under the control of their native promoters. To address this possibility, the plasmid pSL107 was utilized, which contains *virA* and *virG* under the control of the *lac* promoter and *virBp:lacZ.* Introduction of pSL107 into MC4100 also failed to activate expression of the *vir* gene fusion in the presence of AS and IPTG. The observed lack of expression was not due to a lack at expression of *virA* and/or *virG* as both proteins were present at detectable levels by Western blot (data not shown). Since *VirA* and *VirG* were present, the *VirA/VirG* signal transduction mechanism may not be functional in *E. coli.* This possibility was addressed through the use of pSY215 which contains a constitutively active $virG^{con}$ under the control of the *lac* promoter and *vtrBp::lacZ.* The $virG^{con}$ is able to activate expression of the *virBp::lacZ* fusion *in A. tumefaciens* strains independent of *virA* and acetosyringone (17, 31, 45). When pSY215 was introduced into MC4100, once again no expression of the *virBp::lacZ* fusion was observed. This suggested that additional genes from *A. tumefaciens* may be required or that *E. coli* may contain specific repressors of *vir* gene expression.

To determine if additional genes from *A. tumefaciens* were required, a cosmid library constructed from chromosomal DNA of a Ti plasmidless strain A136, was introduced into MC4100(pSY215). Screening of the resulting transformants revealed the presence of a clone which produced a light blue color on colonies grown on induction media containing X-Gal and IPTG. This cosmid clone, designated pBK2, contains a 25 Kb DNA insert. In order to identify the gene residing in pBK2 required for expression of *virBp::lacZ,* overlapping subclones of pBK2 were generated which were introduced into MC4100(pSY215). Expression of the fusion was detected following introduction of pBX4.1, but not pBKS7.0 or pBKE4.8 (Fig. 1 & Table 2). The inability of pBKS7.0 and pBKE4.8 to activate expression of the fusion indicated that the 0.55 Kb region between

these two subclones is required for expression of the fusion. Given this information, a 1.3 Kb StuI-PstI DNA fragment from pBX4.1 was isolated and subcloned it into pTZ18R and pTZ19R yielding pPS1.3R and. PPSI.3, respectively. This resulted in two constructs in which the *lac* promoter on the vector drives transcription from either end of the fragment. Expression of the *vir* fusion was detected only with pPS1.3, indicating the absence of promoter elements in the fragment and the responsible gene is in the direction of *PstI* to SruI. Subclone pPS1.3 resulted in the highest level of expression of the fusion, almost 15 fold higher than pBK2, and 40 fold higher than the vector control. This could be due to both increased copy number of the gene and increased gene expression by the strong *lac* promoter (Table 2).

Characterization of the identified gene

The DNA sequence of the entire 1.3 Kb *StuI-PstI* fragment was obtained from both strands. Analysis of the DNA sequence revealed an open reading frame of 1,008 base pairs, in the predicted transcriptional direction, sufficient to encode a polypeptide of 336 amino acids as shown in Figure 2A. Nucleotide and protein searches of GenBlank and Swiss-Prot databases indicated a high degree of similarity to *rpoA* genes, encoding the α subunit of RNAP. Comparison with *rpoA* from *E. coli* indicated 62.15% sequence similarity and 51.4% sequence identity at the amino acid level as shown in Figure 3. Three highly conserved regions can be identified from the distribution of homologous amino acid residues between *E. coli* and *A. tumefaciens rpoA* homologues. One region extends from residue 30 to 51 near the N-terminus (20 of 22 identical), with the other two present in the C-terminal domain extending from residue 256 to 270 (13 of 15 identical) and from residue 276 to 315 (30 of 42 identical). A notable difference is the presence of an additional eight residues at the C-terminus of the *A. tumefaciens* RpoA, compared to RpoA of *E. coli*. *A* potential Shine-Dalgamo sequence, GAAGGT, was found extending from -7 to -12 bp upstream of the proposed ATG initiation codon of *rpoA*. Analysis of partial DNA sequence obtained from pBKS7.0 and pBKE4.8 indicated the presence of regions upstream and downstream of *rpoA* with a high degree of sequence similarity to *rpsK* and *rplQ*, encoding S11 and L17 ribosomal proteins, respectively, possibly forming an operon structure similar to *E. coli*.

Confirmation of the ORF was achieved by construction of a His-tagged *rpoA* fusion as described above, which yielded a polypeptide of the predicted size (~ 37 Kd) that is

slightly larger than *E. coli* RpoA (Fig. 4A). Furthermore, the DNA clone from *A. tumefaciens* was able to complement a temperature sensitive *rpoA* mutant of *E. coli*, HN317, proving that it does encode a functional homologue of the RNAP α subunit.

In vitro transcription of the *virB* promoter

In vitro assays involving the *rpoA* subclones and a constitutive $virG^{con}$, *in vitro* transcription assays were performed. Purified individual components of *E. coli* RNAP, β β' and σ^{70}, were mixed with either His-RpoA of *E.coli* or *A. tumefaciens* and high molecular weight RNAP complexes were purified by sizing column (see Materials and Methods). As shown in Figure 4A, both RpoA molecules were able to successfully assemble with the *E. coli* β β' and σ^{70} subunits into complete RNAP holoenzymes. When tested for *in vitro* transcription, both RNAP holoenzymes were equally efficient in initiating transcription from a α^{70} dependent *E. coli ga/P1* promoter (Fig. 4B), demonstrating that the hybrid RNAP containing *RpoA* (α-subunit of RNAP) of *A. tumefaciens* is a functional enzyme. Furthermore, no significant differences were detected in the amount of the transcript produced in the presence or absence *of* $virG^{con}$. When the *virB* promoter was used as a template, the *E. coli* KNAP could activate low level transcription but no difference was evident with or without the $VirG^{con}$ protein. In contrast, the hybrid RNAP containing *A. tumefaciens* RpoA was able to activate transcription from the *virB* promoter at low levels, and addition of the $VirG^{con}$ increased transcription by 4-5 fold as measured by quantification of the gel (Fig. 4B). These results confirm the *in* vivo assays demonstrating that only RNAP containing RpoA of *A. tumefaiens* is able to efficiently initiate transcription from the *virB* gene promoter in a $VirG^{con}$ dependent manner. The inability of *E. coli* RNAP to efficiently express the *vir* fusion even in the presence of the $VirG^{con}$ suggests that *E. coli* RpoA can not make the required contacts with the $VirG^{con}$ protein.

Specific interaction between the $VirG^{con}$ and RpoA of *A. tumefaciens*

Since the C-terminal domain of the *E. coli* RpoA was known to interact with the A+T rich "UP element of certain promoters (Ross 1993), the *E. coli* and *A. tumefaciens* RpoA proteins were tested to determine if these proteins have different affinities for the *virB* promoter. As shown in Figure 5 (lanes 3, 4 and *5), A. tumefaciens* RpoA was able to shift the mobility of the labeled *virB* promoter at the highest concentration used (600nM), whereas the same concentration of *E. coli* RpoA did not, suggesting that the RpoA of *A. tumefaciens* has a higher affinity for the *virB* promoter than RpoA of *E. coli*. Similar

mobility shift assays were carried out to determine if His-RpoA of E. coli and A tumefaciens interact differently with VirG^{con} at the *virB* promoter. Increasing amounts of RpoA were used in. combination with a saturating quantity of VirG^{con} for the *virB* promoter (Fig. 5, lane 2) The concentration of VirG^{con} used was determined through separate mobility shift assays in which increasing amounts of VirG^{con} resulted in two separate shifts in mobility, corresponding to binding of VirG^{con} at the two *vir* boxes of the *virB* promoter (data not shown). At the three concentrations of *E. coli* RpoA used, there was no additional shift in the mobility of the promoter VirG^{con} complex (Fig. 5A, lanes 6. 7 and 8). However, when RpoA from *A. tumefaciens* was used, two separate shifts were observed (Fig 5B, lanes 6, 7 and 8) which suggests specific interactions between VirG^{con} and RpoA from *A. turnefaciens*. These results provide further evidence suggesting that RpoA from *A. turnefaciens* may possess a higher affinity for VirG^{con} compared to RpoA from *E. coli*.

Combination of *virA/G* and *rpoA of A.* tumefaciens is insufficient to reconstitute acetosyringone mediated *vir* gene induction in E. coli

To determine if the signaling mechanism, resulting in *vlr* gene activation, can be reconstituted in *E. coli*, a wild type v*irA* and *virG* was used in combination with *A. turnefaciens rpoA*. M04100 harboring two plasmid constructs, one containing a v*irBp::lacZ* fusion as well as wild type *virA/virG* under their native promoters (pGP159), and. the other containing a *lac*-driven *A. tumefaciens rpoA* gene (pH098), did not show *any* significant increase in β-galactosidase activity in the presence of acetosyringone (data not shown). The possibility that lack of expression of *virA* and/or *virG* may account for this result was again addressed through the use of pSL107 which contains *lac-driven virA/virG* and *virBp::lacZ.* However, it was not possible to obtain significant expression of the fusion in MC4100 harboring pSL107 and pH098. Introduction of pH098 into MC4100(pLG2), containing *lac-driven VirG^{con}* and *virBp::lacZ,* however, resulted in a significant increase in β-galactosidase activity, demonstrating that pH098 is able to produce a functional RpoA protein. These results suggest that the signal transduction mechanism of VirA/VirG may not be functional in *E. coli.*

The present invention discloses that it is possible to obtain the expression of heterologous genes that previously could not have been expressed in a host, such as *E. coli*, by including a *rpoA* gene or a portion thereof from the same host genus as the expressed gene is obtained The ability to use *E. coli* as a heterologous system provides investigators

with a valuable tool for studying these various processes. The identification of a chromosomally encoded *A. tumefaciens rpoA* gene and the demonstration that it constitutes one of the components required for expression of a *virBp::lacZ* gene fusion in a heterologous *E. coli* background supports the present invention. The *rpoA* gene of *E. coli* has been extensively studied, particularly with regard to interactions with transcriptional regulators, and suggests that interaction between VirG and RpoA may be required for efficient transcription of virulence genes.

The inability of pGP159 or pSL107 to activate transcription of the *virBp::lacZ* fusion suggested that either signal transduction between VirA and VirG was not functional, or that additional gene(s) were required from *A. tumefaciens* for activation. The use of pSY215 containing a virG^{con} mutant allowed us to evaluate *vir* gene expression in a *virA*-independent manner, eliminating the need for signal transduction. The lack of expression obtained with pSY215 in *E. coli,* combined with its ability to function in a Ti plasmidless *A. tumefaciens* strain suggested that additional *A. tumefaciens* gene(s) were required for expression. The introduction of pPS1.3 containing *lac* driven rpoA into MC4100(pST215) resulted in a significant increase (40 fold) in transcription of the *virBp::lacZ* fusion compared to the control vector. Verification that *rpoA* is required was obtained through the use of subclone pPS1.3R, which did not activate expression of the fusion. This construct is identical to pPS1.3, but the direction of transcription of rpoA is opposite to that of the *lac* promoter. The observation that *A. tumefaciens rpoA* was able to complement a temperature sensitive rpoA mutant in *E. coli* demonstrates an ability to function at essential *E. coli* promoters. This is evident from the in vitro transcription assay where the hybrid NAP was equally effective as *E. coli* RNAP in transcribing a σ^{70}-dependent *ga/P1* promoter (Fig. 4B). While the expression of the *virBp::lacZ* fusion (Table 1) was significantly increased, the level of expression was relatively low in comparison to expression *in A. tumefaciens* (31). The relatively low expression of the *virBp::lacZ* fusion may have been a consequence of the presence of RNAP containing RpoA of *E. coli*. In order to remove possible interference from *E. coli* RpoA, *in vitro* transcription assays using reconstituted RNAP holoenzymes containing His-RpoA from either *E. coli* or *A. tumefaciens* were carried out. Using purified *E. coli* β, β' and α^{70} subunits, it was demonstrated that both of the His-RpoA were able to assemble into multi-subunit RNAP holoenzymes (Fig. 4A). The results of the *in vitro* transcription assays demonstrated that VirG^{con}-dependent transcription of the

virBp::lacZ fusion requires RNAP containing *A. tumefaciens* RpoA, although the two reconstituted holoenzymes exhibited essentially identical activity in transcription from the ga/P1 promoter, with no significant difference in the presence or absence of $VirG^{con}$ (Fig 4B). Another possible explanation for the relatively low induction in *E. coli* may due to the presence of *E. coli* sigma factors in the RNAP holoenzymes. It is conceivable that *E. coli* sigma factors have a lower affinity for the virB promoter than sigma factors from *A. turnefaciens*. Although the vegetative sigma factor from *A. turnefaciens* has been identified (50), it *is* unclear whether this or an alternative sigma factor is involved in *vir* gene transcription.

Previous reports have identified the presence of an "UP element" in certain *E. coli* promoters which is required for optimal transcription (14, 41, 47). This element extends from -40 to -60 bp upstream of the transcription start site and is highly A+T rich. Interestingly, the *virB* promoter contains an A+T rich sequence from -40 to -60 that overlaps wit the VirG binding sites. Whether this region of the promoter constitutes a true "UP element" is unknown. From the gel shift assays, the *A. tumefaciens* RpoA appears to have a higher affinity for the *virB* promoter than *E. coli* RpoA (Fig 5A and B), although the importance of this observation is unclear at this time. The results of the mobility shift assay suggest that *E. coil* RpoA is unable to bind to $VirG^{con}$ at the *virB* promoter. In contrasts, *A. tumefaciens* RpoA appears to exhibit cooperative binding with two distinct shifts in the mobility of the $VirG^{con}$-*virB* promoter complex. Taken together, these results indicate that RNAP containing *E. coli* RpoA may be unable to interact effectively with $VirG^{con}$, and therefore can not activate transcription from the *virB* promoter. Since the *virB* promoter contains two binding sites for VirG, the presence of two shifts obtained with increasing amounts of *A. tumefaciens* RpoA may be a consequence of RpoA interacting with VirG at each *vir* box.

The two component regulatory system, composed of *vir*A and *vir*G, is indispensible for transcription of virulence genes within *A. tumefaciens*. However, *vir*A and *vir*G are insufficient to activate transcrition from virulence gene promoters within *E. coli* cells, indicating a requirement for additional *A. tumefaciens* gene(s).

In examining *vir* gene expression in *E. coli,* attempts to reconstitute wild type virulence gene expression in *E. coli* were not successful. The use of *virA* and *virG* under the control of the *lac* promoter means that sufficient levels of *vir*A and *vir*G should be

present for signal transduction to take place. One possible explanation may be that *E. coli* is unable *to* correctly insert *vir*A into the inner membrane. Alternatively, even, though *vir*A may be inserted into the inner membrane correctly, dimerization of *vir*A which is required for activity in. *A. turnefuciens* (43) may be defective. A more likely explanation may be that additional gene(s) from *A. tumefaciens* are required for efficient signal transduction. An unresolved question is the exact mechanism of sensing of phenolic inducers by the *vir*A/*vir*G system. The two possible mechanisms involve direct binding of the inducer by *vir*A, or binding by a second receptor which then interacts with *vir*A. Although genetic evidence supporting direct binding of inducers by *vir*A has been reported (36, 37), all attempts to demonstrate direct binding by *vir*A have been unsuccessful. Conversely, there have been reports in which binding of phenolic compounds by proteins other than *vir*A have been detected (13, 35), although there is no evidence to link these proteins with *vir* gene induction. The search for additional *A. tumefaciens* gene(s) involved in the signal transduction should be simplified by determining that *vir*G^{con} mediated expression of virulence genes requires RpoA from *A. tumefaciens*. The present invention provides the basis to examine *vir* gene expression as well as the T-DNA transfer process in *E. coli*.

This specific example shows that for the expression of *vir* genes in *E. coli*, both *rpoA* and *virG* from *A. tumefaciens* are required for transcriptional activation of a *vir* promoter in *E. coli*. It has been determined that the *rpoA* gene, encoding the α subunit of RNAP, confers significant expression of a virBp;;lacZ fusion in *E.coli* in the presence of an active transcriptional regulator *vir*G gene. *In vitro* transcription assays were conducted using either reconstituted *E. coli* RNAP or hybrid RNAP in which the α subunit was derived from *A. tumefaciens*. Both RNAPs were equally efficient in transcription from a σ^{70}- dependent *E. coli* galP1 promoter, however, only the hybrid RNAP was able to transcribe the *vir*B promoter in a *vir*G-dependent manner.

Evidence is also presented which indicates that *vir*G interacts with rpoA from *A. tumefaciens* but not with rpoA from *E. coli*. This observation suggests that in order for successful transcription of *vir* genes to occur, specific interaction(s) between the *A. tumefaciens* α subunit of RNAP and *vir*G is required.

As shown in Figure 3, one of the major difference between the RpoA sequences of *A. tumefaciens* and *E.coli* is the presence of an extra 8 consecutive amino acids at the C-

terminus of *A. tumefaciens* RpoA (amino acids 329 to 336). To test if these C-terminal 8 consecutive amino acids are important in mediating *vir* gene activation in *E.coli*, a plasmid construct was generated from pPS1.3 by deleting the DNA coding for the last 8 amino acids using site directed mutagenesis. The resulting plasmid pAD8 encodes 328 amino acid long *A. tumefaciens* RpoA, lacking the original amino acids from 329 to 336.

The *vir* gene activating ability of this construct was tested by introducing pAD8 into *E.coli* strain MC4100 harboring pSY215 which contains *virA*, *virG* and *virB::lacZ* fusion. As negative and positive controls, vector plasmid pQE31 or pPS1.3 which encodes intact RpoA of *A. tumefaciens* was introduced into MC4100(pSY215), respectively. The *vir* gene activation was monitored by measuring the β-galactosidase activities. As shown in Table 3, pAD8 failed to activate β-galactosidase activity whereas the pPS1.3 induced a high level β-galactosidase activity. These results indicate that the C-terminal 8 amino acids of *A. tumefaciens* RpoA are essential for the *vir* gene activation in *E.coli*.

These experiments also demonstrate that incorporation of a *rpoA* gene of a prokaryote, such as *A.tumefaciens*, into an expression vector will promote the expression of genes from the prokaryotic cells in other prokaryotic cells, such as *E. coli*. Expression of this expression vector comprising at least a portion of an *A. tumefaciens* gene is a useful gene delivery system in heterologous hosts for gene delivery and gene therapy. Further, incorporation of at least a portion of an *rpoA* gene derived from the same cells as one or more heterologous genes, into a vector containing the heterologous genes, provide improved systems for expressing the heterologous genes. These expression vectors which comprise one or more heterologous genes and at least a portion of an *rpoA* gene derived from the same cells as the heterologous genes, are useful in the production of selected metabolites, by expression genes for an entire metabolic pathway in a heterologous host.

The following list of publications referred to in the specification are herein incorporated in their entirety by reference.

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Table 1. Bacterial Strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Source
E. coli		
DH5α	*recA endAI hsdR17 supE4 gyrA96 reLAI (lacZYA-argF)U169 (80dlacZ M15)*	(56)
MC4100	F`araD139 (argF-kac)U169 rpsL150 relA fib5301 ptsF25deoC1	(57)
HN317	rpoA112^{ts}St^{r}	(58)
Plasmids		
pGP159	*virA. virG & virBp*::lacZ; IncP, Ap^{r},TC^{r}	(59)
pSM234acd	*virA & virBp*::lacZ: IncP. Km^{r}	(45)
pLG2	*lacp*::*virG*(N54D) & *virBp*::lacZ; IncP, Ap^{r}Tc^{r}	This study
pSL107	*lacp*::*virG* & *vir*Bp::*lacZ*:IncW,AP^{r}Tc^{r}	This study
pPC401(N54D)	*lacp*::*virG*(N54D) in pTZ18R.Ap^{r}	This study
pSY215	*virBp*::lacZ & *lacp*::virG(N54D); IncW, Gm^{r}	This study
pVK102	Cosmid cloning vector; IncP, Km^{r},Tc^{r}	(60)
pTZ18R/pTZ19R	Cloning vector; ColEl.Ap^{r}	USB
pBK2	pVK102 with 25 Kb chromosomal insert from A.*tumefaciens strain* A136, Tc^{r}	This Study
pBKS2-2	pTZ18R with 7.1 Kb *Sau*3A insert from pBK2	This study
pBX4.1	pTZ18R with 4.3 Kb *Bam*HI-XbaI insert from pBKS2-2	This study
pBKS7.0	pTZ18R with 4.2 Kb *Sau*3A-Sa*l*I insert from pBKS2-2	This study
pBKE4.8	pTZ18R with 1.7 Kb *Eco*RI-*Sau*3A insert from pBKS2-2	This study
pPS1.3R	pTZ18R with 1.3 Kb *PstI-Stu*I insert from pBKS2-2	This study
pPS1.3	pTZ19R with 1.3 Kb *Pst*I-*Stu*I insert from pBKS2-2	This study
pZL-2	Overproducer of *A. tumefaciens* His-RpoA: Ap^{r}	This study
pECH4	Overproducer of *E. coli* His-RpoA.Ap^{r}	This study
pH098	*lacp*::rpoA of *A. tumefaciens* from pPS1.3: IncW.Km^{r}	This study

Table 2. RpoA mediated expression of *vir*BP::lacZ fusion in *E. coli* MC4100 containing *vir*G[test]

Constructs	LB medium		MG/L medium	
	β-Galactosidase[b]	Fold Activation[c]	β-Galactosidase[b]	Fold Activation
pBK102	2.46	NA	2.35	NA
pBK2	5.2	2.11 X	6.56	2.79 X
pTZ18R	2.24	NA	2.51	NA
pBKS2-2	13.67	6.1 X	7.25	2.89 X
pBX4.1	26.42	11.8 X	8.11	3.23 X
pBKS7.0	1.68	0.75 X	2.46	0.98 X
pBKE4.8	2.36	1.05 X	1.63	0.65 X
pBP3.0	5.45	2.43 X	2.83	1.13 X
pPS1.3R	2.13	0.95 X	1.71	0.68 X
pPS1.3	76.14	40.0 X	37.69	15.0 X

The *virBp*::*lacZ* expression assays were carried out in LB and MG/L medium for 16 hours as described in the text, IPTG was added at a final concentration of 1 mM. *E. coli* MC4100 containing pSY215 (*virG*[con] + *virBp*::lacZ).[b] Values are the average of three replicates.

[c] Values indicate an increase or decrease in activity compared to the appropriate control vector. NA, Not Applicable.

Table 3. β-galactosidase activities

E.coli strain MC4100(pSY215) plus plasmid shown in right	pPS1.3	pAD8	pQE31
β-galactosidase in Miller Units*	140 (+/-25)	4 (+/-1)	3 (+/-2)

* Miller Units = Defined in Ref. 68.

We claim:

1. An isolated nucleic acid molecule encoding at least a portion of the α subunit of an RNAP of *Agrobacterium*.

2. An isolated nucleic acid molecule encoding the complete α subunit of an RNAP of *Agrobacterium*.

3. An isolated nucleic acid molecule encoding the complete α subunit of an RNAP of *Agrobacterium*, wherein the amino acid sequence of said α subunit is depicted in Figure 2A.

4. An isolated nucleic acid molecule encoding at least a portion of the α subunit of an RNAP of *Agrobacterium*, wherein the amino acid sequence of said α subunit is depicted in Figure 2A.

5. The nucleic acid molecule of claim 4, wherein said portion of the amino acid sequence as depicted in Figure 2A comprises at least amino acid residues 157 to 336.

6. The nucleic acid molecule of claim 4, wherein said portion of the amino acid sequence as depicted in Figure 2A comprises less than amino acid residues 157 to 336.

7. The nucleic acid molecule of claim 4, wherein said portion of the amino acid sequence as depicted in Figure 2A comprises at least 8 consecutive amino acid residues in length.

8. The nucleic acid molecule of claim 7, wherein said portion of the amino acid sequence as depicted in Figure 2A comprises amino acid residues 329 to 336.

9. An isolated nucleic acid molecule comprising the nucleic acid sequence as depicted in Figure 2B.

10. An isolated nucleic acid molecule comprising at least a portion of the nucleic acid sequence as depicted in Figure 2B.

11. A hybrid nucleic acid molecule for the expression of at least one heterologous gene in a host cell, wherein said hybrid nucleic acid molecule comprises a first nucleic acid sequence encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the host cell and a second nucleic acid sequence encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the heterologous gene.

12. The isolated nucleic acid molecule of claim 11, wherein said second nucleic acid sequence is obtained from a prokaryotic cell selected from the group consisting of

Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

13 The isolated nucleic acid molecule of claim 12, wherein said second nucleic acid sequence is obtained from the genus *Agrobacterium*, *Mycobacterium*, *Chlamydia*, *Pseudomonas* or *Streptomyces*.

14. The nucleic acid molecule of claim 13, wherein said second nucleic acid sequence is obtained from *Agrobacterium*.

15. The nucleic acid molecule of claim 14, wherein said second nucleic acid sequence encodes the amino acid sequence as depicted in Figure 2A.

16. The nucleic acid molecule of claim 14, wherein said second nucleic acid sequence encodes at least a portion of the amino acid sequence as depicted in Figure 2A.

17. The nucleic acid molecule of claim 16, wherein said portion of the amino acid sequence as depicted in Figure 2A comprises at least amino acid residues 157 to 336.

18. The nucleic acid molecule of claim 16, wherein said portion of the amino acid sequence as depicted in Figure 2A comprises less than amino acid residues 157 to 336.

19. The nucleic acid molecule of claim 16, wherein said portion of the amino acid sequence as depicted in Figure 2A comprises at least 8 consecutive amino acid residues in length.

20. The nucleic acid molecule of claim 19, wherein said portion of the amino acid sequence as depicted in Figure 2A comprises amino acid residues 329 to 336.

21. The hybrid nucleic acid molecule of claim 11, wherein said first nucleic acid sequence is obtained from a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

22. The nucleic acid molecule of claim 21, wherein said first nucleic acid sequence is obtained from the genus *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*.

23. A nucleic acid construct comprising a nucleic acid molecule of any one of claims 1-22 operably linked to a promoter.

24. A vector comprising the nucleic acid construct of claim 23.

25. A host cell comprising a nucleic acid construct of claim 23 or a vector of claim 24, wherein said promoter is operable in said host cell.

26. A host cell of claim 25, further comprising at least one heterolgous gene operably linked to a promoter.

27. A host cell of claim 26, wherein said heterolgous gene and promoter are contained in a vector.

28. A host cell of any one of claims 25-27, wherein said host cell is a prokaryotic cell or a eukaryotice cell.

29. A host cell of claim 28, wherein said host cell is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

30. A host cell of claim 29, wherein said host cell is an *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*.

31. A method of expressing at least one heterologous gene in a host cell comprising:

transforming said host cell with a nucleic acid molecule of 23 and with at least one heterologous gene operably linked to a promoter; and

culturing said transformed host cell under conditions wherein at least one of said heterologous genes is expressed in said host cell.

32. The method of claim 31, further comprising transforming said host cell with at least one gene encoding a heterologous transcriptional regulator obtained from the same genus as the source of the heterologous gene.

33. The method of claim 32, wherein said transcirptional regulator is a transcriptional activator that interacts with the α subunit of the RNAP to enhance the expression of said heterologous gene.

34. The method of any one of claims 31 - 33, wherein said heterologous gene comprises multiple genes or operons in the same metabolic pathway.

35. The method of claim 31, wherein said nucleic acid molecule and said at least one heterologous gene are contained in at least one vector.

36. The method of claim 31, wherein said host cell is transformed with a vector comprising the nucleic acid molecule and with at least one vector comprising at least one heterologous gene.

37. The method of claim 31, wherein said host cell is a prokaryotic cell or a eukaryotic cell.

38. The method of claim 37, wherein said host cell is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fact bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

39. The method of claim 38, wherein said host cell is an *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*.

40. The method of claim 39, wherein said prokaryotic cell is an *Escherichia*.

41. The method of claim 31, wherein said genus of the source of the heterolgous gene is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

42. The method of claim 41, wherein said genus is *Agrobacterium*, *Mycobacterium*, *Chlamydia*, *Pseudomonas* or *Streptomyces*.

43. The method of claim 42, wherein said genus is obtained from *Agrobacterium*.

44. A method of expressing at least one heterologous gene in a host cell comprising:

transforming said host cell with a nucleic acid molecule encoding a complete α subunit of an RNA polymerase obtained from the same genus as the heterologous gene or a portion of said α subunit of the RNA polymerase operably linked to a promoter, and with at least one heterologous gene operably linked to a promoter; and

culturing said transformed host cell under conditions wherein at least one of said heterologous genes is expressed in said host cell.

45. The method of claim 44, further comprising transforming said host cell with at least one gene encoding a heterologous transcriptional regulator obtained from the same genus as the source of the heterologous gene.

46. The method of claim 45, wherein said transcirptional regulator is a transcriptional activator that interacts with said α subunit of the RNAP to enhance the expression of said heterologous gene.

47. The method of any one of claims 44-46, wherein said heterologous gene comprises multiple genes or operons in the same metabolic pathway.

48. The method of claim 44, wherein said nucleic acid molecule and said at least one heterologous gene are contained in at least one vector.

49. The method of claim 44, wherein said host cell is transformed with a vector comprising the nucleic acid molecule and with at least one vector comprising at least one heterologous gene.

50. The method of claim 44, wherein said host cell is a prokaryotic cell or a eukaryotic cell.

51. The method of claim 50, wherein said host cell is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, aicd-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

52. The method of claim 51, wherein said host cell is from the genus *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*.

53. The method of claim 52, wherein said prokaryotic cell is an *Escherichia*.

54. The method of claim 44, wherein said genus of the source of the heterolgous gene is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

55. The method of claim 54, wherein said genus is *Agrobacterium*, *Mycobacterium*, *Chlamydia*, *Pseudomonas* or *Streptomyces*.

56. A purified α subunit of an RNA polymerase of *Agrobacterium* or a portion thereof.

57. A purified α subunit of an RNA polymerase of *Agrobacterium* of claim 55 or a portion thereof wherein the amino acid sequence of said α subunit is depicted in Figure 2A or a portion thereof.

58. A purified α subunit of an RNA polymerase of *Agrobacterium* of claim 55 or portion thereof, wherein the α subunit of RNA polymerase is encoded by the nucleic acid is depicted in Figure 2B or a portion thereof.

AMENDED CLAIMS
[received by the International Bureau on 15 August 2000 (15.08.00);
original claims 3-10, 15-20, 28, 34, 57 and 58 amended;
remaining claims unchanged (6 pages)]

1. An isolated nucleic acid molecule encoding at least a portion of the α subunit of an RNAP of *Agrobacterium*.

2. An isolated nucleic acid molecule encoding the complete α subunit of an RNAP of *Agrobacterium*.

3. An isolated nucleic acid molecule encoding the complete α subunit of an RNAP of *Agrobacterium*, wherein the amino acid sequence of said α subunit is depicted in Figure 2A (SEQ ID NO:1).

4. An isolated nucleic acid molecule encoding at least a portion of the α subunit of an RNAP of *Agrobacterium*, wherein the amino acid sequence of said α subunit is depicted in Figure 2A (SEQ ID NO:1).

5. The nucleic acid molecule of claim 4, wherein said portion of the amino acid sequence as depicted in Figure 2A (SEQ ID NO:1) comprises at least amino acid residues 157 to 336.

6. The nucleic acid molecule of claim 4, wherein said portion of the amino acid sequence as depicted in Figure 2A (SEQ ID NO:1) comprises less than amino acid residues 157 to 336.

7. The nucleic acid molecule of claim 4, wherein said portion of the amino acid sequence as depicted in Figure 2A (SEQ ID NO:1) comprises at least 8 consecutive amino acid residues in length.

8. The nucleic acid molecule of claim 7, wherein said portion of the amino acid sequence as depicted in Figure 2A (SEQ ID NO:1) comprises amino acid residues 329 to 336.

9. An isolated nucleic acid molecule comprising the nucleic acid sequence as depicted in Figure 2B (SEQ ID NO:2).

10. An isolated nucleic acid molecule comprising at least a portion of the nucleic acid sequence as depicted in Figure 2B (SEQ ID NO:2).

11. A hybrid nucleic acid molecule for the expression of at least one heterologous gene in a host cell, wherein said hybrid nucleic acid molecule comprises a first nucleic acid sequence encoding at least a portion of the α subunit of an RNAP obtained from the same genus as the host cell and a second nucleic acid sequence encoding at least a

portion of the α subunit of an RNAP obtained from the same genus as the heterologous gene.

12. The isolated nucleic acid molecule of claim 11, wherein said second nucleic acid sequence is obtained from a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

13 The isolated nucleic acid molecule of claim 12, wherein said second nucleic acid sequence is obtained from the genus *Agrobacterium*, *Mycobacterium*, *Chlamydia*, *Pseudomonas* or *Streptomyces*.

14. The nucleic acid molecule of claim 13, wherein said second nucleic acid sequence is obtained from *Agrobacterium*.

15. The nucleic acid molecule of claim 14, wherein said second nucleic acid sequence encodes the amino acid sequence as depicted in Figure 2A (SEQ ID NO:1).

16. The nucleic acid molecule of claim 14, wherein said second nucleic acid sequence encodes at least a portion of the amino acid sequence as depicted in Figure 2A (SEQ ID NO:1).

17. The nucleic acid molecule of claim 16, wherein said portion of the amino acid sequence as depicted in Figure 2A (SEQ ID NO:1) comprises at least amino acid residues 157 to 336.

18. The nucleic acid molecule of claim 16, wherein said portion of the amino acid sequence as depicted in Figure 2A (SEQ ID NO:1) comprises less than amino acid residues 157 to 336.

19. The nucleic acid molecule of claim 16, wherein said portion of the amino acid sequence as depicted in Figure 2A (SEQ ID NO:1) comprises at least 8 consecutive amino acid residues in length.

20. The nucleic acid molecule of claim 19, wherein said portion of the amino acid sequence as depicted in Figure 2A (SEQ ID NO:1) comprises amino acid residues 329 to 336.

21. The hybrid nucleic acid molecule of claim 11, wherein said first nucleic acid sequence is obtained from a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

22. The nucleic acid molecule of claim 21, wherein said first nucleic acid sequence is obtained from the genus *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*.

23. A nucleic acid construct comprising a nucleic acid molecule of any one of claims 1-22 operably linked to a promoter.

24. A vector comprising the nucleic acid construct of claim 23.

25. A host cell comprising a nucleic acid construct of claim 23, wherein said promoter is operable in said host cell.

26. A host cell of claim 25, further comprising at least one heterologous gene operably linked to a promoter.

27. A host cell of claim 26, wherein said heterologous gene and promoter are contained in a vector.

28. A host cell of claim 26, wherein said host cell is a prokaryotic cell or a eukaryotic cell.

29. A host cell of claim 28, wherein said host cell is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

30. A host cell of claim 29, wherein said host cell is an *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*.

31. A method of expressing at least one heterologous gene in a host cell comprising:

transforming a host cell with a nucleic acid molecule of 23 and with at least one heterologous gene operably linked to a promoter; and

culturing said transformed host cell under conditions wherein at least one of said heterologous genes is expressed in said host cell.

32. The method of claim 31, further comprising transforming said host cell with at least one gene encoding a heterologous transcriptional regulator obtained from the same genus as the source of the heterologous gene.

33. The method of claim 32, wherein said transcriptional regulator is a transcriptional activator that interacts with the α subunit of the RNAP to enhance the expression of said heterologous gene.

AMENDED SHEET (ARTICLE 19)

34. The method of claim 31, wherein said heterologous gene comprises multiple genes or operons in the same metabolic pathway.

35. The method of claim 31, wherein said nucleic acid molecule and at least one of said heterologous genes are contained in at least one vector.

36. The method of claim 31, wherein said host cell is transformed with a vector comprising the nucleic acid molecule and with at least one vector comprising at least one heterologous gene.

37. The method of claim 31, wherein said host cell is a prokaryotic cell or a eukaryotic cell.

38. The method of claim 37, wherein said host cell is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fact bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

39. The method of claim 38, wherein said host cell is an *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*.

40. The method of claim 39, wherein said prokaryotic cell is an *Escherichia*.

41. The method of claim 31, wherein said genus of the source of the heterologous gene is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

42. The method of claim 41, wherein said genus is *Agrobacterium*, *Mycobacterium*, *Chlamydia*, *Pseudomonas* or *Streptomyces*.

43. The method of claim 42, wherein said genus is obtained from *Agrobacterium*.

44. A method of expressing at least one heterologous gene in a host cell comprising:

transforming said host cell with a nucleic acid molecule encoding a complete α subunit of an RNA polymerase obtained from the same genus as the heterologous gene or a portion of said α subunit of the RNA polymerase operably linked to a promoter, and with at least one heterologous gene operably linked to a promoter; and

culturing said transformed host cell under conditions wherein at least one of said heterologous genes is expressed in said host cell.

45. The method of claim 44, further comprising transforming said host cell with at least one gene encoding a heterologous transcriptional regulator obtained from the same genus as the source of the heterologous gene.

46. The method of claim 45, wherein said transcriptional regulator is a transcriptional activator that interacts with said α subunit of the RNAP to enhance the expression of said heterologous gene.

47. The method of any one of claims 44-46, wherein said heterologous gene comprises multiple genes or operons in the same metabolic pathway.

48. The method of claim 44, wherein said nucleic acid molecule and said at least one heterologous gene are contained in at least one vector.

49. The method of claim 44, wherein said host cell is transformed with a vector comprising the nucleic acid molecule and with at least one vector comprising at least one heterologous gene.

50. The method of claim 44, wherein said host cell is a prokaryotic cell or a eukaryotic cell.

51. The method of claim 50, wherein said host cell is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

52. The method of claim 51, wherein said host cell is from the genus *Escherichia*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Clostridium* or *Rhizobium*.

53. The method of claim 52, wherein said prokaryotic cell is an *Escherichia*.

54. The method of claim 44, wherein said genus of the source of the heterologous gene is a prokaryotic cell selected from the group consisting of Gram-negative bacteria, Gram-positive bacteria, acid-fast bacteria, mycoplasma, aerobic bacteria, anaerobic bacteria, and facultative bacteria.

55. The method of claim 54, wherein said genus is *Agrobacterium*, *Mycobacterium*, *Chlamydia*, *Pseudomonas* or *Streptomyces*.

56. A purified α subunit of an RNA polymerase of *Agrobacterium* or a portion thereof.

57. A purified α subunit of an RNA polymerase of *Agrobacterium* of claim 55 or a portion thereof wherein the amino acid sequence of said α subunit is depicted in Figure 2A (SEQ ID NO:1) or a portion thereof.

58. A purified α subunit of an RNA polymerase of *Agrobacterium* of claim 55 or portion thereof, wherein the α subunit of RNA polymerase is encoded by the nucleic acid is depicted in Figure 2B (SEQ ID NO:2) or a portion thereof.

FIG. 1

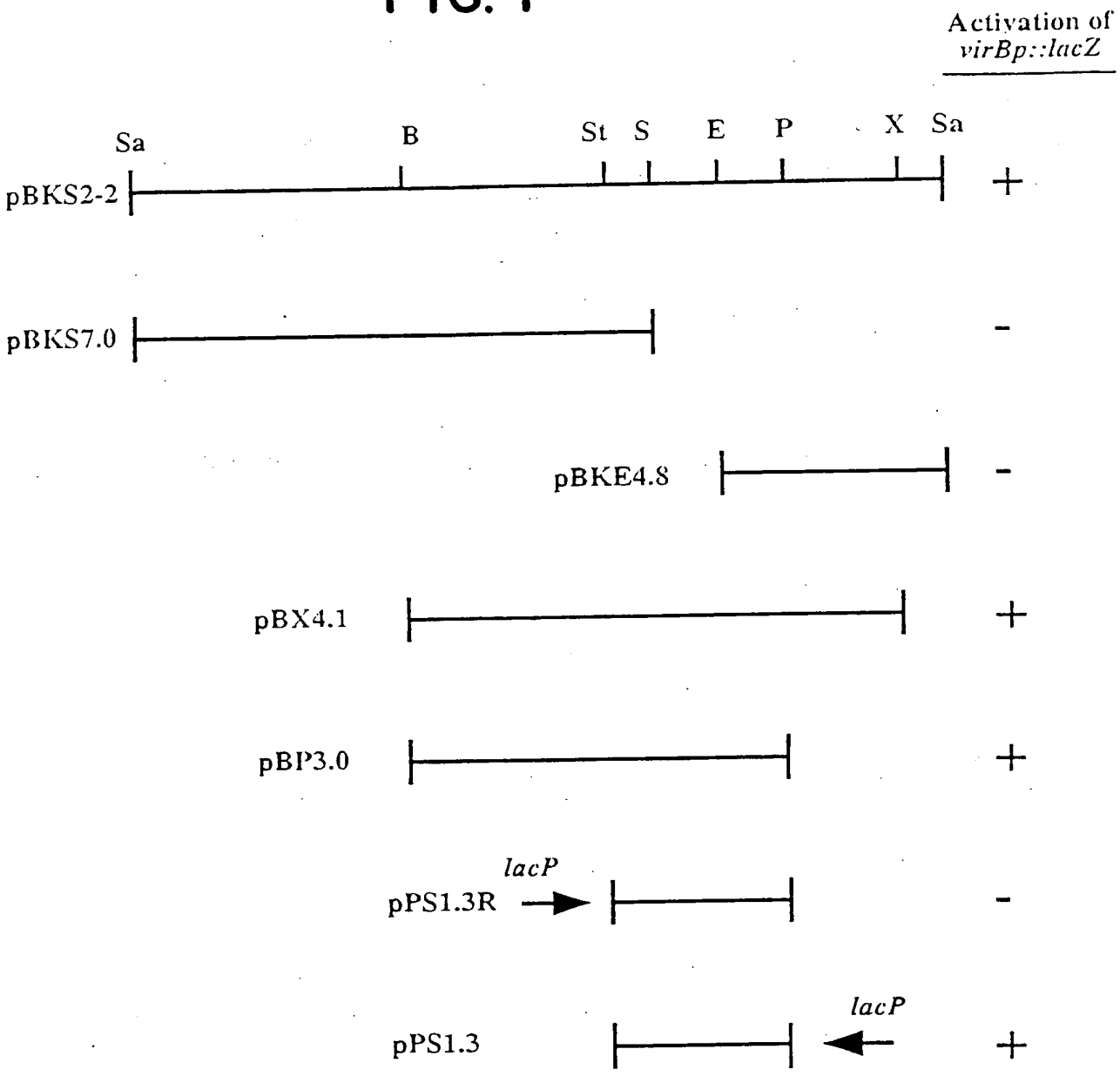


FIG. 2A

MIQKNWQELIKPNKVEFTSSSRTKATLVAEPLERGFGLTLGNALRRVLLS	50
SLRGAAVTAVQIDGVLHEFSSIPGVREDVTDIVLNIKEIAIKMDGDDSKR	100
MVVRKQGPGSVTAGDIQTVGDIEILNPDHVICTL.DEGAEIRMEFTVNNG	149
KGYVPAE---RNRAEDAPIGLIPVDSLYSPVKKVSYKVENTREGQVLDYD	196
KLIMTIETNGSVSGEDAVAFAARILQDQLGVFVNFDEPQKEAEEESVTEL	246
AFNPALLKKVDELELSVRSANCLKNDNIVYIGDLIQKTEAEMLRTPNFGR	296
KSLNEIKEVLASMGLHLGMEVPAWPPENIEDLAKRYEDQY	336

FIG. 2B

1 aacacggcat gaagtcgctt gaagtcgaag tttgcggtcc gggttccggt cgtgaatcgg
61 cacttcgcgc tctgcaggct gccggtttca tgatcacttc cattcgcgac gccgatcccg
121 cacaacggtt gccgtccgcg caagaagcgc cgcgtctgac gcgaccgtgg ttcggaaatt
181 ccgcctttcc ttcggtctgg cggaattttc gtgtatctgg cgtgtgcgcg tcgatttcga
241 tcgacggact tgcgctcaag aacccactga tgaaccactg aattaggttc ctctcggtgt
301 tttcatgctc ggtccgtcac gattggatgg tggcggcgaa cggaaggttt aaagatgatt
361 cagaagaact ggcaggaact tatcaagccg aacaaggtcg agttcacctc gtccagccgc
421 accaaggcaa ctctggttgc cgagccgctg gagcgtggtt tcggtcttac cctcggcaac
481 gcgctgcgcc gcgttctgtt gtcttctctg cgtggtgccg ctgtaacggc cgtgcagatc
541 gacggtgtcc tgcacgaatt ctcctccatc cccggcgttc gggaagatgt gacggatatc
601 gtgctcaaca tcaaggaaat cgccatcaag atggatggtg acgattccaa gcgcatggtc
661 gtgcgcaagc agggtccggg ttcggtaacc gctggtgaca tccagacggt tggcgacatc
721 gagatcctga accccgacca cgtgatctgc acgctcgatg aaggcgctga aatccgcatg
781 gaattcaccg tcaacaacgg caagggttac gtaccggctg agcgcaaccg cgcggaagat
841 gcccctatcg gcctcattcc ggtggacagc ctctattctc cggtcaagaa agtgtcctac
901 aaggtggaaa acacccgtga aggtcaggtt ctcgactatg acaagctgat catgacgatc
961 gagaccaacg gttcggtttc cggcgaagac gccgttgcct tcgccgctcg cattcttcag
1021 gaccagctgg gcgtcttcgt caacttcgac gagccgcaga aggaagcaga agaagaatcg
1081 gttactgaac tcgcgttcaa cccggcgctt ctcaagaagg tcgacgagct cgaactgtca
1141 gttcgttcgg caaactgcct gaagaacgac aacatcgttt atatcggcga cctgatccag
1201 aagaccgaag ccgaaatgct ccgcacgccg aactttggtc gcaagtcgct gaacgaaatc
1261 aaggaagttc tcgcttccat gggtctgcac ctcggcatgg aagtgccggc atggccgcct
1321 gagaacatcg aagatctcgc aaagcgttac gaagaccaat actaacaaac aagaaggcag
1381 accttaaaga ctgcctttcc ccgtcaaaca gcagataagt catctgcatg tgccaggaaa
1441 cggcaggcct taaagaaggc acctgcgtag aaggagaata gcaatgcgcc acggtaattc
1501 aggccgcaag ctcaatagaa ccgccagcca ccgcaaggca atgtttgcca acatggctgc
1561 ttcgctcatc acccatgagc agatcgtcac cacccttccg aaggcgaagg aaatccgtcc
1621 gatcgtcgag cgtctcgtga cgctgggcaa gcgcggcgac ctgcacgctc gtcgtcaggc
1681 gatctcgcag at

FIG. 3

```
A. tum.   MIQKNWQELIKPNKVEFTSSSRTKATLVAEPLERGFGLTLGNALRRVLLS 50
E. coli   M-QGSVTEFLKPRLVDIEQVSSTHAKVTLEPLERGFGHTLGNALRRILLS 49
          * * .  * .**  *.    * * * .  ******** ********.***

A. tum.   SLRGAAVTAVQIDGVLHEFSSIPGVREDVTDIVLNIKEIAIKMDGDDSKR 100
E. coli   SMPGCAVTEVEIDGVLHEYSTKEGVQEDILEILLNLKGLAVRVQGKDEVI 99
          *. * *** *.*******.*.  **.**. .*.**.* .*... * *

A. tum.   MVVRKQGPGSVTAGDIQTVGDIEILNPDHVICTL.DEGAEIRMEFTVNNG 149
E. coli   LTLNKSGIGPVTAADITHDGDVEIVKPQHVICHLTDENASISMRIKVQRG 149
          . . * * * *** **   **.**. * **** * ** * * *   *  *

A. tum.   KGYVPAE---RNRAEDAPIGLIPVDSLYSPVKKVSYKVENTREGQVLDYD 196
E. coli   RGYVPASTRIHSEEDERPIGRLLVDACYSPVERIAYNVEAARVEQRTDLD 199
          .*****     .  .. *** . **. ****....* **  *  *  * *

A. tum.   KLIMTIETNGSVSGEDAVAFAARILQDQLGVFVNFDEPQKEAEEESVTEL 246
E. coli   KLVIEMETTGTIDPEEAIRRAATILAEQLEAFVDLRDVRQPEVKEEKPE- 248
          **.. .** *..  *.*.  ** ** .**  **.  . ..   .*   *

A. tum.   AFNPALLKKVDELELSVRSANCLKNDNIVYIGDLIQKTEAEMLRTPNFGR 296
E. coli   -FDPILLRPVDDLELTVRSANCLKAEAIHYIGDLVQRTEVELLKTPNLGK 297
           *.* **. **.***.******** . * *****.*.** *.*.*** *.

A. tum.   KSLNEIKEVLASMGLHLGMEVPAWPPENIEDLAKRYEDQY 336
E. coli   KSLTEIKDVLASRGLSLGMRLENWPPASIADE 329
          *** ***.**** ** ***  .  ***  .* *
```

FIG. 4B

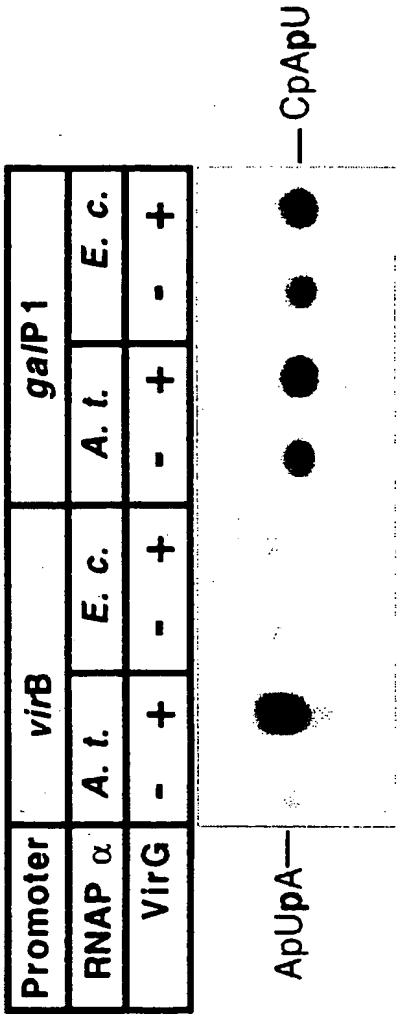


FIG. 4A

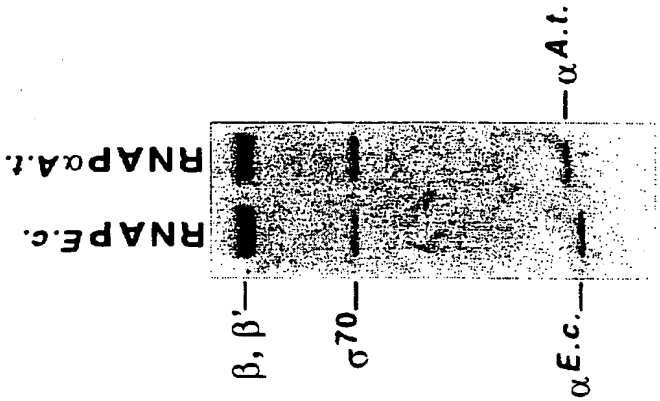


FIG. 5A

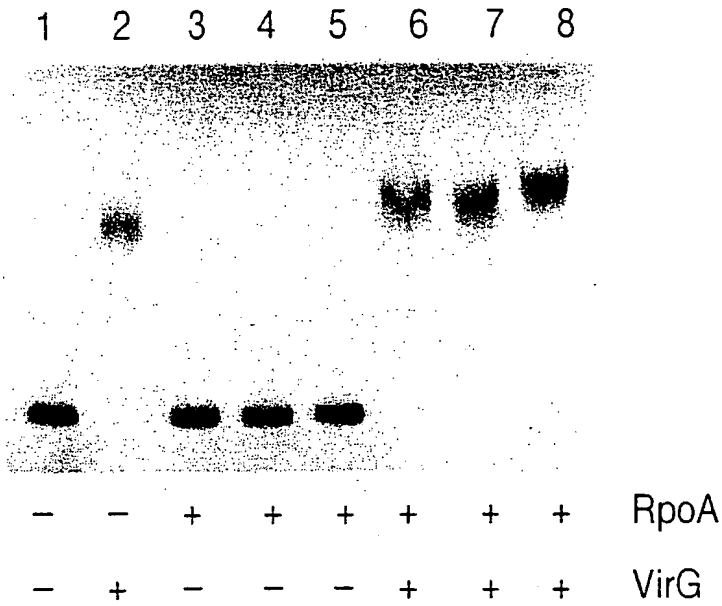
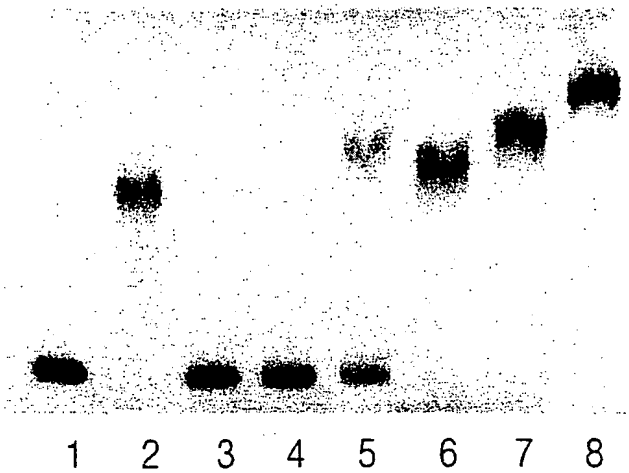


FIG. 5B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/10014

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12P 21/02; C12N 15/62; C07H 21/04; C12N 9/00
US CL :435/69.1, 183; 536/23.1, 23.4

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 183; 536/23.1, 23.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	LOHRKE et al. Transcriptional Activation of *Agrobacterium tumefaciens* Virulence Gene Promoters in *Escherichia coli* Requires the *A. tumefaciens* rpoA Gene, Encoding Alpha Subunit of RNA Polymerase. Journal of Bacteriology. August 1999, Vol. 181, No. 15, pages 4533-4539.	1, 2, 11-14, 21-24, 31-33, 35-56
A	STEFFEN et al. Hybrid *Bordetella pertussis-Escherichia coli* RNA Polymerases: Selectivity of Promoter Activation. Journal of Bacteriology. March 1998, Vol. 180, No. 6, pages 1567-1569.	11-14, 21-24, 31-33, 35-56

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 14 JUNE 2000	Date of mailing of the international search report 20 JUL 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer GERALD G. LEFFERS JR. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/10014

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BOYLAN et al. Gene Encoding the Alpha Core Subunit of *Bacillus subtilis* RNA Polymerase is Cotranscribed with the Genes for Initiation Factor 1 and Ribosomal Proteins B, S13, S11, and L17. Journal of Bacteriology. May 1989, Vol. 171, No. 5, pages 2553-2562, especially Figure 2.	11-14, 21-24, 31-33, 35-56
A	WO 98/24891 A1 (SCRIPTGEN PHARMACEUTICALS, INC.) 11 June 1998 (11.06.98), see entire document, especially pages 2-4.	11-14, 21-24, 31-33, 35-56

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/10014

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 3-10, 15-20, 57-58
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

No sequence listing or computer readable form of the sequence listing was submitted with this case. Also, the claims are drawn to sequences given in figures rather than to sequences given in a sequence listing and having sequence identifiers.

3. ☒ Claims Nos.: 25-30, 34
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/10014

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

EAST: DERWENT, JPO, EPO, USPAT; STN: MEDLINE, EMBASE, BIOSIS, CAPLUS
search items: inventor names, rpoA, map, rna polymerase, alpha subunit, hybrid, fusion, agrobacterium, tumefaciens, coli, escherichia, pseudomnas, bacilus, lactobacillus, rhizobium, mycobacterium, chlamydia, streptomyces